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**14. ABSTRACT**

Hyper-activation of the PI3K-AKT-mTOR signaling pathway is frequently observed in advanced prostate cancer and has been suggested to promote survival of prostate cancer cells under androgen deprivation. The transcription factor C/EBP beta is expressed as three different translational isoforms from a single transcript and has been suggested to regulate mTOR activity. The longer LAP isoforms promote cell survival, growth arrest and differentiation whereas the LIP isoform can promote cell proliferation. The purpose of these studies were to evaluate the role of C/EBP beta translational isoforms in prostate cancer resistance to chemotherapy. The 4E/G interaction inhibitor (4E/Gi), which blocks cap-dependent translation, significantly upregulated the LAP isoforms in both PC3 and LNCaP cells. Suppression of C/EBP beta in PC3 cells increased mTOR activity and induction of LAP expression by 4E/Gi and bicalutamide treatment additively suppressed mTOR. We also evaluated the proteasome inhibitor bortezomib as another means to alter C/EBP isoforms, because it can also inhibit cap-dependent translation. Bortezomib increased the LAP:LIP ratio in LNCaP and PC3 cells and suppression of CEBPB sensitized these cells to bortezomib *in vitro.* PC3 xenografts deficient in CEBPB showed suppressed growth and were also sensitized to bortezomib administration. Our data suggest that CEBPB LAP isoforms suppress AKT-mTOR activity, but may protect prostate cancer cells from bortezomib. Conversely, increasing CEBPB LAP isoform levels by blocking cap-dependent translation may be an effective strategy to prevent transition to castrate-resistant prostate cancer or sensitize prostate cancer cells to mTOR inhibitors by suppressing AKT activity.

**15. SUBJECT TERMS**

mTOR, PI3K, C/EBP beta, androgen receptor, cap-dependent translation

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## 1. Introduction:

Central to the survival of prostate cancer (PCa) are the androgen receptor (AR) and phosphatidylinositol-3 kinase (PI3K)-AKT signaling pathways. Indeed, it has been reported that 50-70% of human prostate cancers have mutations in PI3K signaling, often through loss of phosphatase and tensin homolog (PTEN). AR gene amplification is also frequently observed in hormone refractory prostate cancer (HRPC). Combined blockade of PI3K and AR signaling is a strong apoptotic stimulus for PCa cells and shows promise for future therapy [1, 2]. Intriguingly, AR and PI3K-AKT signaling are reciprocal inhibitors of one another, but can both promote cell survival and proliferation in PCa. Inhibition of a single pathway leads to alternative survival by the other. PI3K-AKT signaling activates mammalian target of rapamycin (mTOR) activity, a critical regulator of cell growth and global protein synthesis. mTOR is often hyper-activated in human cancer due to high metabolic demand of rapidly growing tumors. mTOR is a kinase that forms two distinct complexes in the cell referred to as mTOR complex (mTORC) 1 and -2. mTORC1 is a complex between mTOR, raptor and LST8, which functions to negatively regulate autophagy and promote protein synthesis [3]. mTORC2 is comprised of mTOR, rictor, LST8 and SIN1, whose best characterized functions are activating AKT and actin cytoskeletal organization [4]. Although a promising therapeutic target, rapamycin and rapalogs have only achieved modest clinical benefit, indicating PCa cells initiate compensatory mechanisms for survival even during decreased AR and mTOR signaling [5, 6]. Members of the CCAAT/enhancer binding protein (C/EBP) family are regulated through translation mechanisms by mTOR activity and have been linked to PCa survival and metastatic gene expression [7, 8]. C/EBPα and C/EBPβ (C/EBPα/β) are both transcription factors that function to exert cell cycle control and terminal differentiation. More recently, these factors have been recognized to suppress both the intrinsic and extrinsic apoptotic pathways through the expression of MCL-1, cFLIP, BCL-2 and BCL-xL [7, 9]. Both members are expressed as a single intronless transcript and truncated translational isoforms are generated by leaky ribosomal scanning. Activity of mTORC1, eukaryotic initiating factor 4E (eIF4E) or eIF2α causes the ribosome to skip the first AUG and initiate translation of the truncated isoforms [8, 10, 11]. Importantly, it has been shown that truncated and long C/EBPα/β isoforms have differential cellular function. Truncated C/EBPs lack transcriptional activation domains and suppress gene transcription by heterodimerizing with full-length isoforms or directly binding to target gene promoters and recruiting HDACs. the long isoforms activate transcription in most contexts and are more functionally linked with survival, cell cycle arrest and terminal differentiation. [10, 12, 13]. In contrast, truncated isoforms function to increase cell growth and act as silencers of tumor suppressor pathways. For example, mutation of the CEBPα gene locus, so that only the truncated p30 isoform is expressed, results in development of acute myeloid leukemia [15]. C/EBPβ LIP expression increases mammary epithelial cell proliferation and antagonizes anti-proliferative signals from TGF-β in breast cancer cells [16]. Because truncated C/EBPs antagonize the activity of full length isoforms, blocking LIP or p30 translation with mTOR or cap-dependent translation inhibitors could promote full length C/EBP translation as a mechanism for PCa cells to suppress proliferation, but avoid apoptosis, e.g. by increasing anti-apoptotic gene expression. The purpose of this research project is to determine the functional roles of C/EBPα/β translational isoforms in PCa progression to CRPC, tumor growth and survival and resistance to androgen deprivation.

## 2. Keywords:

mTOR, PI3K, C/EBP beta, androgen receptor, cap-dependent translation

## 3. ACCOMPLISHMENTS

- **What were the major goals of the project?**
  - The overall objective of of this fellowship award is to train and develop the career of Dr. Barakat so that he can function as an independent prostate cancer investigator. The research specific goals of the award period are as follows:
    1. Evaluate C/EBPα/β translational isoform expression in PCa cell lines treated with pharmacological inhibitors of the PI3K-AKT-mTOR signaling pathway. Months 1-8. 100% completed.
    2. Evaluate proliferation and survival in PCa cell lines with combined C/EBPα/β KD or over-expressing individual C/EBP translational isoforms. Months 9-13. 75% completed.
    3. Mouse studies with engineered prostate cancer cell lines. Months 13-24. Not completed.

  - Training-specific goals for this award period were as follows:
    1. Attend selected prostate cancer specific research seminars. 100% completed

2. Attend 2015 American Association for Cancer Research annual meeting. Completed April 2015.
3. Present ongoing research at Prostate Cancer Research Day. Completed February 2015.
4. Attend the AACR Translational Cancer Research for Basic Scientists workshop. Not completed. My submitted application for this workshop was rejected. I have since re-applied for the 2015 workshop and have been put on a waiting list for attendance.
5. Publication of CCAAT/Enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells. **Barakat DJ**, Zhang J, Barberi T, Denmeade SR, Friedman AD, Paz-Priel I. Oncogene. 2015 Mar 16. doi: 10.1038/onc.2015.41. Completed November 2014.
6. Submit K99/R00 Pathway to Independence award. Completed.

- **What was accomplished under these goals?**

1. Major Activities:
   The major research activities for this award period included the investigation of the role of the signaling pathways that regulated C/EBPβ translation isoform expression, the role of C/EBPβ in regulating AKT-mTOR activity and resistance to mTOR inhibitors and androgen deprivation.

   Training specific activities included attendance and poster presentation at the 2015 American Association for Cancer Research national meeting in Philadelphia. This work was also presented as a poster presentation at two internal Johns Hopkins events: Prostate Cancer Research day and Fellows Research day. I also submitted a K99/R00 NIH pathway to independence grant. This task was originally planned for months 12-24, but had to be completed during the first year of the award because the NIH had changed the eligibility criteria. Postdoctoral fellows with more than 4 years of training by the time the award is reviewed are ineligible to apply. In the beginning months of the award, I published an article in the journal Oncogene titled: CCAAT/Enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells. This work was submitted prior to the fellowship award start date and a revision was completed during the award period.

2. The specific objectives for this award period were the training and educational development of Dr. Barakat in prostate cancer research, determination of the critical regulators of C/EBPβ translational isoform expression in prostate cancer cells and evaluating the proliferation and survival of prostate cancer cells deficient in C/EBP transcription factors in response to chemotherapy.

3. Significant Results:
   C/EBPα and -β are transcription factors that are expressed from an intronless transcript. Truncated isoforms, C/EBPα p30 and C/EBPβ LIP, which lack transcriptional activation domains are generated by translation from in-frame, down-stream start codons and can exert dominant-negative effects by heterodimerizing with full-length isoforms [8]. Because previous studies had indicated that cap-dependent translation and mTOR were critical drivers of C/EBPα/β truncated isoforms, we therefore evaluated the effect of pharmacological inhibitors of PI3K, mTOR or cap-dependent translation on C/EBP translational isoforms by Western blot analysis. We treated the PTEN-null LNCaP and PC3 cell lines for 6 hours with PI3K inhibitor Ly294002 (25 μM), mTORC1 inhibitor RAD001 (100nM), mTORC1/2 inhibitor INK128 and 4E/Gi (25 μM), which blocks cap-dependent translation by disrupting the interaction between eukaryotic initiation factor 4E (eIF4E) and eIF4G. In LNCaP and PC3 cells, blockade of mTOR or PI3K lead to down regulation of all C/EBP isoforms. Intriguingly, treatment with 4E/Gi lead to induction of C/EBPβ LAP isoforms in LNCaP cells with preferential induction of LAP. Further, 4E/Gi increased expression of all three isoforms in PC3 cells (figure 1a). Expression of C/EBPα was almost undetectable in LNCaP cells and modest in PC3 cells, indicating that it may not play a strong role in response to mTOR inhibition in prostate cancer cells. Blocking PI3K or mTOR decreased phosphorylation of p70S6K in LNCaP cells, but had no effect in PC3 cells. Intriguingly, TORC1/2 blockade with INK128 blocked phosphorylation of eIF4E in both lines, indicating that cap-dependent translation is suppressed. We next evaluated C/EBPα/β gene expression under similar conditions in these cell lines by quantitative real-time PCR (qPCR) to determine whether the changes in C/EBP expression were attributable to altered mRNA

levels. Blockade of mTOR by RAD001 or INK128 lead to significant down-regulation of C/EBPα/β gene expression and 4E/Gi had no effect on expression of these genes in LNCaP cells (Fig 1B). Ink128 down-regulated C/EBPβ levels and 4E/Gi modestly up-regulated CEBPB transcript levels in PC3 cells (Fig. 1B). These results indicate that cap-dependent translation rather than mTOR activity plays a crucial role in regulating the LIP:LAP isoform ratio in prostate cancer cell lines. Because we found that C/EBPα expression was minimally expressed in these cell lines, we focused our efforts on C/EBPβ for the remaining studies.

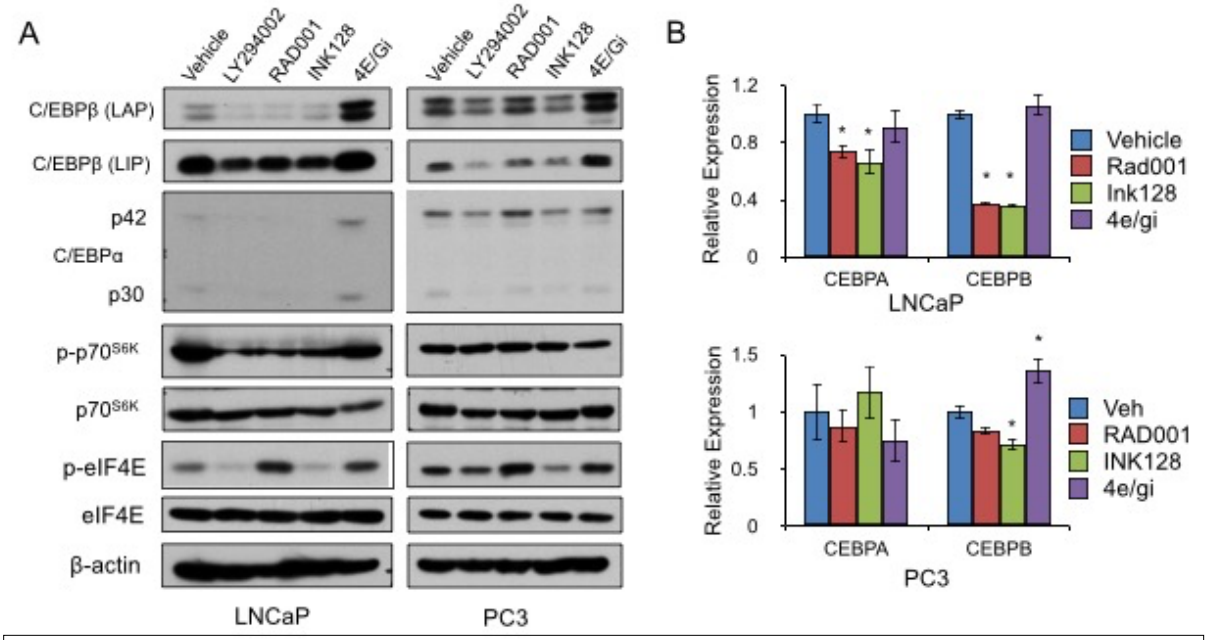


**Figure 1: Cap-dependent translation promotes C/EBP full-length isoform expression in PTEN-null prostate cancer cell lines.** Western blot **(A)** and qPCR **(B)** analysis of PC3 and LNCaP cells treated for 6 hours with 25μM LY294002, 100nM RAD001, 100nM INK128 or 50μM 4E/Gi. Bar graphs represent average of three experiments. Error bars: SEM. *p<0.01.

Although mTOR activity did not have a selective effect on C/EBPβ translational isoforms, we did observe an overall decrease in C/EBPβ protein levels in LNCaP and PC3 cells. Further, it has been suggested that C/EBPβ can regulate the activity of AKT-mTOR signaling by regulating REDD1 expression {Jin 2009}. These observations suggest that expression of C/EBPβ by mTOR activity could function in a negative feedback loop. We therefore evaluated whether C/EBPβ could regulate mTOR activity in PCa cells by shRNA knockdown and ectopic expression. These two lines are useful for evaluating the function of LIP and LAP because they show differential expression of these isoforms. We stably transduced PC3 cells with pTRIPZ-shRNA lentiviral vectors with doxycycline-inducible expression of scrambled RNA or shRNA targeting *CEBPB* and evaluated cell lysates for phosphorylation status of p70S6K as a readout for mTOR activity. After 72 hours of treatment with 0.25μg/ml doxycycline, we observed a substantial increase in phosphorylated p70S6K (Fig. 2A, left). Conversely, we generated PC3 cell lines with stably integrated doxycycline-inducible piggybac vectors for ectopic expression of C/EBPβ. We incubated cells with 0.5μg/ml doxycycline for 72 hours and evaluated cell lysates for phosphorylation status of p70S6K. Unexpectedly, the total levels of p70S6K were suppressed, but phosphorylation status of p70S6K remained unchanged (Fig 2A, right). Activation of mTOR activity in PC3 cells deficient in *CEBPB* was correlated with an decrease in REDD1 gene expression (Fig. 2B). Knockdown of CEBPB in LNCaP cells increased REDD1 expression 2-fold, likely owing to predominant LIP expression in this cell line (Fig. 2B and see Barakat et al., Fig 4A). To determine whether loss of C/EBPβ could influence prostate cancer response to mTOR inhibitors, we treated PC3 or LNCaP shCEBPB cells with escalating doses of the mTOR ATP-site inhibitor Ink128. We found that suppression of C/EBPβ decreased sensitivity of PC3 cells to Ink128 and had no effect on LNCaP cells (Fig 2C). These data suggest that C/EBPβ LAP promotes sensitivity to INK128 and suppresses mTOR activity in PC3 cells.

Androgen deprivation can increase activity of mTOR to promote survival of prostate cancer cells and tumors [1, 2]. However, combined rapamycin treatment with androgen deprivation did not achieve clinical benefit [5]. We have previously shown that anti-androgens dose dependently increase CEBPB and transcript and protein levels (see Barakat et al., 2015 figure 2). However, the LAP:LIP isoform ratio remained low under these conditions suggesting that cap-dependent translation remains high under conditions of androgen deprivation. Because blocking cap-dependent translation with 4E/Gi increased LAP expression in LNCaP cells and LAP expression suppresses mTOR activity, we next evaluated whether combined blockade of androgen receptor (AR) signaling in combination with 4E/Gi could increase the LAP:LIP isoform ratio than either treatment alone and suppress mTOR activity. We treated LNCaP cells with vehicle, 25μM bicalutamide, 50μM 4E/Gi or their combination for 24 hours. As expected, combined treatment of 25μM bicalutamide with 25μM 4E/Gi increased LAP:LIP isoform ratio to a greater extent than 4E/Gi alone (figure 2D). This also lead to a greater decrease in p70S6K phosphorylation and greater increase in REDD1 expression. These results suggest that manipulating C/EBPβ translational isoforms to favor LAP or decrease LIP translation could be a means to suppress mTOR activity during androgen deprivation. These data also have implications for C/EBPβ as a driver of resistance to hormonal therapy.

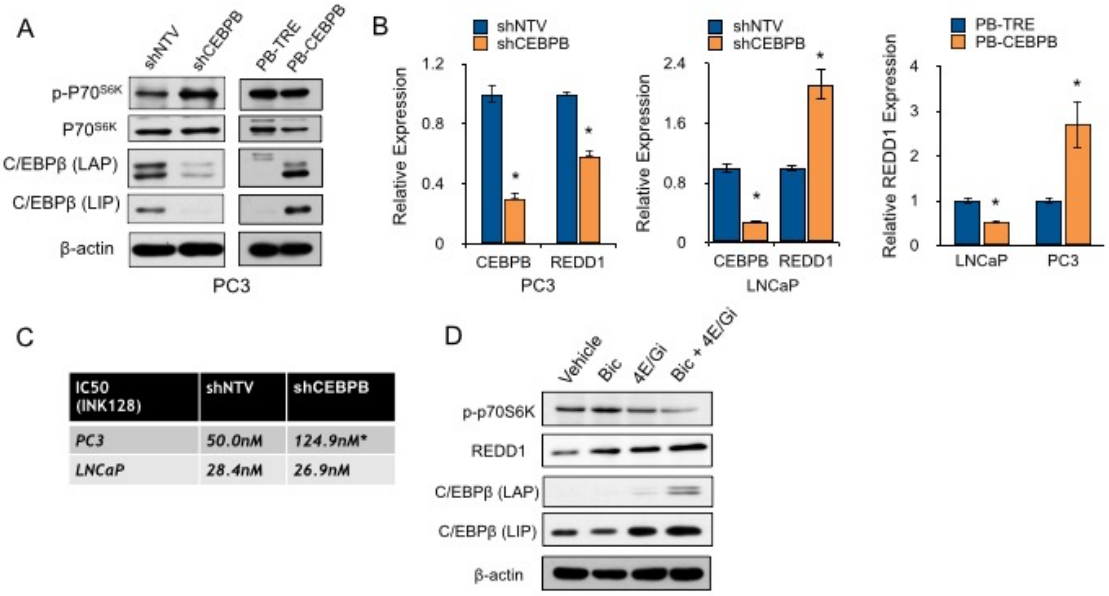


**Figure 2. C/EBPβ LAP suppresses mTOR and promotes REDD1 expression. (A)** Western blot analysis of PC3 cells expressing shRNA targeting *CEBPB* or ectopically expressing mouse C/EBPβ. **(B)** qPCR analysis of REDD1 expression in PC3 and LNCaP cells with *CEBPB* KD or ectopic C/EBPβ expression (right-most bar graph). **(C)** Western blot analysis of LNCaP cells lysates from cultures treated with vehicle (DMSO 1:1000), 25μM bicalutamide (Bic), 50μM 4E/Gi or their combination (Bic + 4E/Gi) for 24 hours. **(D)** IC50 values derived from from WST-1 assay of shCEBPB PC3 and LNCaP cells treated with escalating doses of INK128 for 48hrs. Bar graphs represent average of three experiments. Error bars: SEM. *p<0.01.

To determine whether C/EBPβ levels correlate with human prostate cancer progression we interrogated the Oncomine database. In the Grasso et al data set (26) that included gene expression patterns from 28 benign prostate tissues, 59 localized PC, and 35 CRPC samples CEBPB expression was significantly (p=1.9x10-6) elevated in CRPC compared with localized disease (Fig. 3A). Because C/EBPβ up regulation was associated with progression to CRPC, we next tested whether C/EBPβ played a role in the development of castrate-resistant growth of PCa tumors in a mouse xenograft model. shNTV or shCEBPB LNCaP cells were subcutaneously engrafted into male NSG mice and when tumors reached a volume between 100 and 300mm$^3$ animals were put on a doxycycline-laced animal feed and surgically castrated seven days later. Tumor volume was monitored weekly by caliper measurement for 8-weeks (Fig. 3B). We observed significant suppression of CRPC growth in xenografts expressing shC/EBPβ (p<0.001). These results suggest that C/EBPβ is a

critical determinant of CRPC growth and that androgen deprivation only modestly increases the LAP:LIP C/EBPβ isoform ratio.

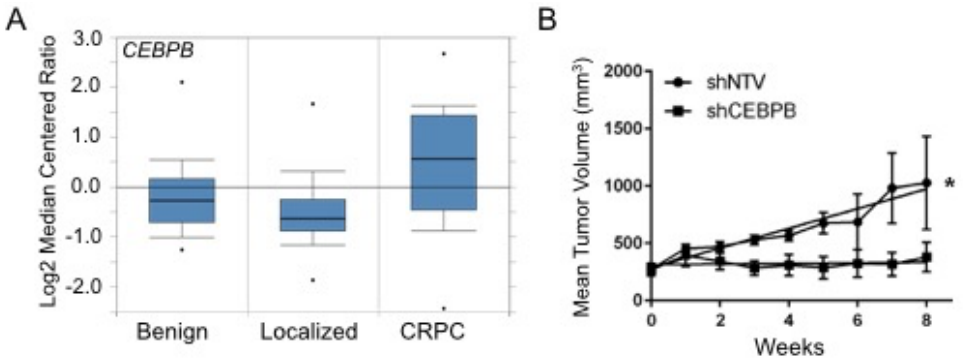


**Figure 3. Suppression of C/EBPβ is critical for CRPC growth. (A)** Relative expression levels of CEBPB in RNA isolated from benign prostate (0), localized prostate cancer (1) and those with heavily treated castrate-resistant prostate cancer (2). Graph obtained from Oncomine using microarray data from Grasso et al., 2012; PMID: 22722839. **(B)** Tumor volumes recorded by caliper measurement in NOG mice following surgical castration. Tumor growth was analyzed by linear regression (* p<0.001).

Because the 4E/Gi did not induce cell death as a single agent in PC3 cells (not shown), we decided to evaluate the effects of other chemotherapy drugs which could potentially suppress cap-dependent translation, alter C/EBPβ translational isoform ratio and promote cell death. The proteasome inhibitor bortezomib promotes inhibition of cap-dependent translation by dephosphorylation of eIF4E binding protein 1 [22]. Further, it has been shown that bortezomib can up-regulate C/EBPβ gene and protein expression [27]. We therefore tested whether bortezomib could alter the C/EBPβ LAP:LIP isoform ratio in PCa cell lines. 24 hours after treatment, we observed up regulation of C/EBPβ transcript and proteins levels in both LNCaP and PC3 cells (Fig 4A and B). Further, there was a dramatic increase in the LAP isoform levels in both of these lines (Fig 4A). We also observed increased expression of REDD1, a suppressor of AKT and mTOR in PC3 cells 48 hours after treatment with 25nM bortezomib (Fig. 4B). These data demonstrate that bortezomib can increase the C/EBPβ LAP:LIP translational isoform ratio in prostate cancer cell lines similar to 4E/Gi.

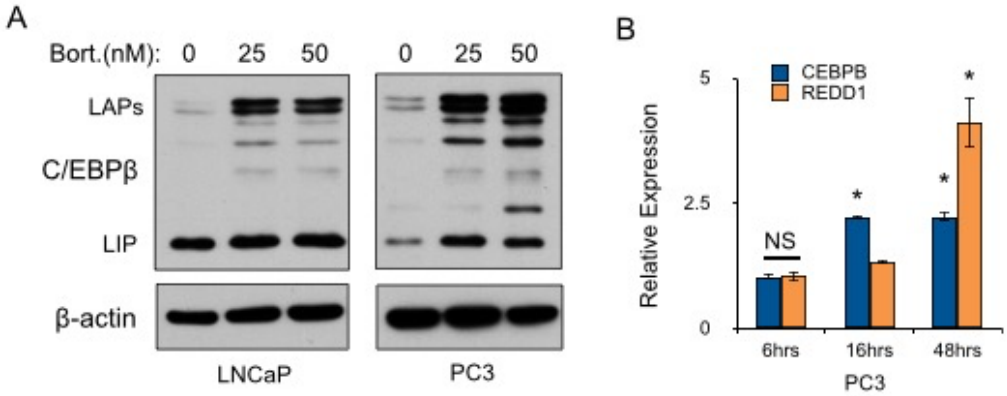


**Figure 4. Bortezomib increases C/EBPβ LAP isoforms in PCa cell lines. (A)** Western blot analysis of C/EBPβ in LNCaP and PC3 cells treated with escalating doses of bortezomib for 24 hours. **(B)** Analysis of *REDD1* and *CEBPB* gene expression in PC3 cells treated with 25nM bortezomib for the indicated times by qPCR. Bar graphs represent average of three experiments. Error bars: SEM. *p<0.01.

We next evaluated the role of C/EBPβ in regulating prostate cancer survival in response to bortezomib. shNTV and shCEBPB LNCaP cells were seeded in 96-well plates and treated with escalating doses of bortezomib for 48 hours. We evaluated cell viability by WST-1 assay and observed a significant decrease in the IC50 value of cells deficient in C/EBPβ indicating increased sensitivity to bortezomib (Fig. 5A). We also evaluated PC3 cells under

similar conditions, but did not observe a significant difference in IC50 value (Fig. 5B). However, this assay may not have been suitable for determining drug sensitivity in these cells because values from treated cells are normalized to vehicle treated controls and we observed a substantial decrease in PC3 growth upon suppression of C/EBPβ (fig 6a and b). This would suggest that the response to drug treatment is actually greater than normalized value in the cells lacking C/EBPβ. We therefore evaluated cell death by trypan blue exclusion in cells treated with 25 and 50nM bortezomib for 24 hours. We observed a significant increase in cell death in shCEBPB cells treated with 25nM (17.2% vs 34.5%) and 50nM (22.8% vs 45.8%) bortezomib (Fig 5C). We further evaluated BCL2 levels in PC3 cells treated with bortezomib and found that BCL2 levels declined rapidly in shCEBPB cells, whereas BCL2 levels in control cells remained constant (Fig 5D). These results demonstrate that C/EBPβ promotes resistance to bortezomib in prostate cancer cells.

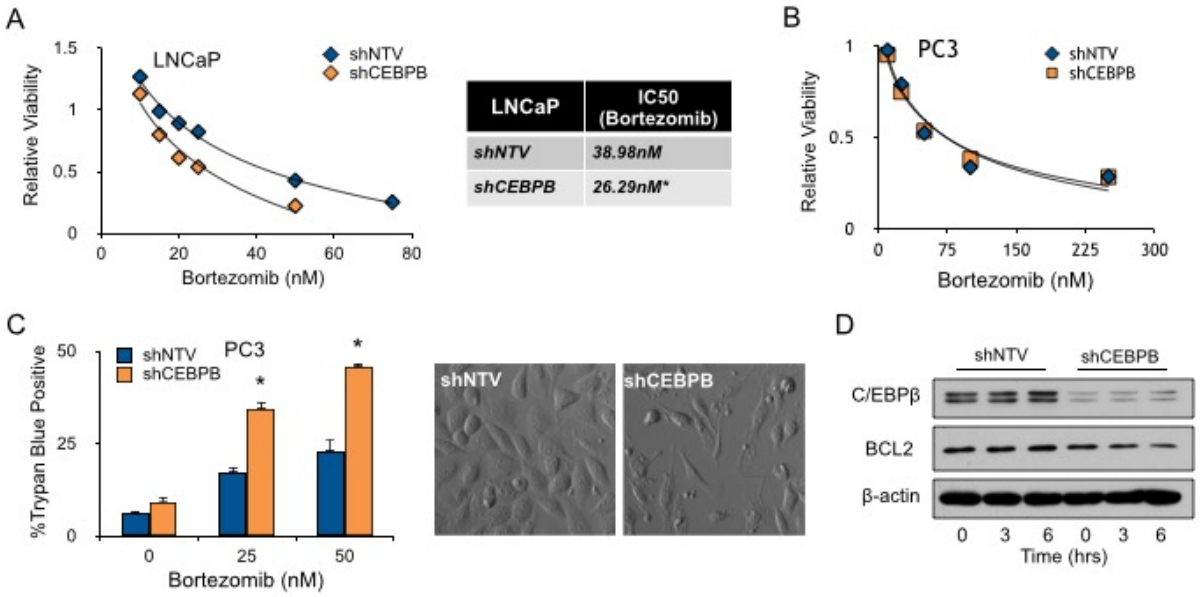


**Figure 5. Suppression of C/EBPβ promotes cell death in response to bortezomib in prostate cancer cell lines. (A and B)** Analysis of PCa cell sensitivity to bortezomib by WST-1 assay in LNCaP and PC3 cells treated with escalating doses of bortezomib for 48 hours. **(C)** Quantification of PC3 death by trypan blue exclusion in cultures treated for 24 hours with 0, 25 and 50nM bortezomib. **(D)** Western blot analysis of BCL2 expression in PC3 cultures treated with 25nM bortezomib for 0, 3 and 6hrs. Bar graphs represent average of three experiments. Error bars: SEM. *p<0.01.

Next, we generated a pair of TALEN expression vectors with targeting sequences to the human CEBPB gene locus and a donor plasmid containing CEBPB homology arms flanking a hygromycin resistance cassette. The region between the homology arms within the CEBPB gene contains the TALEN targeting sites. We transfected PC3 cells with these vectors and 48hrs later added hygromycin (250µg/ml) to the medium. Cells were incubated for 4 days in hygromycin before being split into 96-well plates for hygromycin resistant sub-clones. Genomic DNA from several subclones were analyzed by PCR with primers designed to amplify the deleted region of CEBPB. We identified one sub-clone which was negative for amplification of this region. However, we were still able to detect C/EBPβ protein in lysates collected from this subline (not shown). A control subline, which was not treated with hygromycin and still showed amplification of the CEBPB region by PCR was utilized as control (TALEN control). Subsequent attempts to generate cell lines with complete knockout were unsuccessful. However, these cells with partial loss of C/EBPβ may still be useful for the study of CEBPB in prostate cancer.

We next characterized the growth rates of TALEN and shCEBPB knockdown cells relative to their respective controls. We evaluated cell growth by manually counting cells with a hemacytometer 2 and 4 days after plating and found that suppression of C/EBPβ dramatically decreased the rate of PC3 cell growth by 2.5-fold in shCEBPB and >3-fold in TALEN KD cells. Consistent with this finding, we also evaluated clonogenic growth and observed a decrease in colony number in cultures deficient in C/EBPβ (fig 7b). Loss of C/

EBPβ has been implicated in cell cycle defects and activation of DNA damage response (refs). We next evaluated DNA content by PI staining in shCEBPB and TALEN KD PC3 cells 72hours after seeding cells in growth medium. Suppression of CEBPB by either method yielded a significant decline in the percentage of G1 cells (Fig. 7C). However, knock down of CEBPB by TALEN targeting also showed an increase in G2/M and aneuploid (>G2/M) population. A similar pattern was observed in shCEBPB cells, but to a lesser extent (Fig 7C). Previous studies have shown cell cycle defects and DNA damage in cells deficient in C/EBPβ [24, 26]. Next, we quantified the number of cells with DNA double strand breaks in shCEBPB cultures using phosphorylated histone 2A.X (γH2A.X) immunofluorescent staining and found a significant, >2-fold increase in cells displaying >5 DNA damage foci (Fig. 7D). These results indicate that C/EBPβ promotes cell proliferation and suppresses DNA damage in PC3 cells.

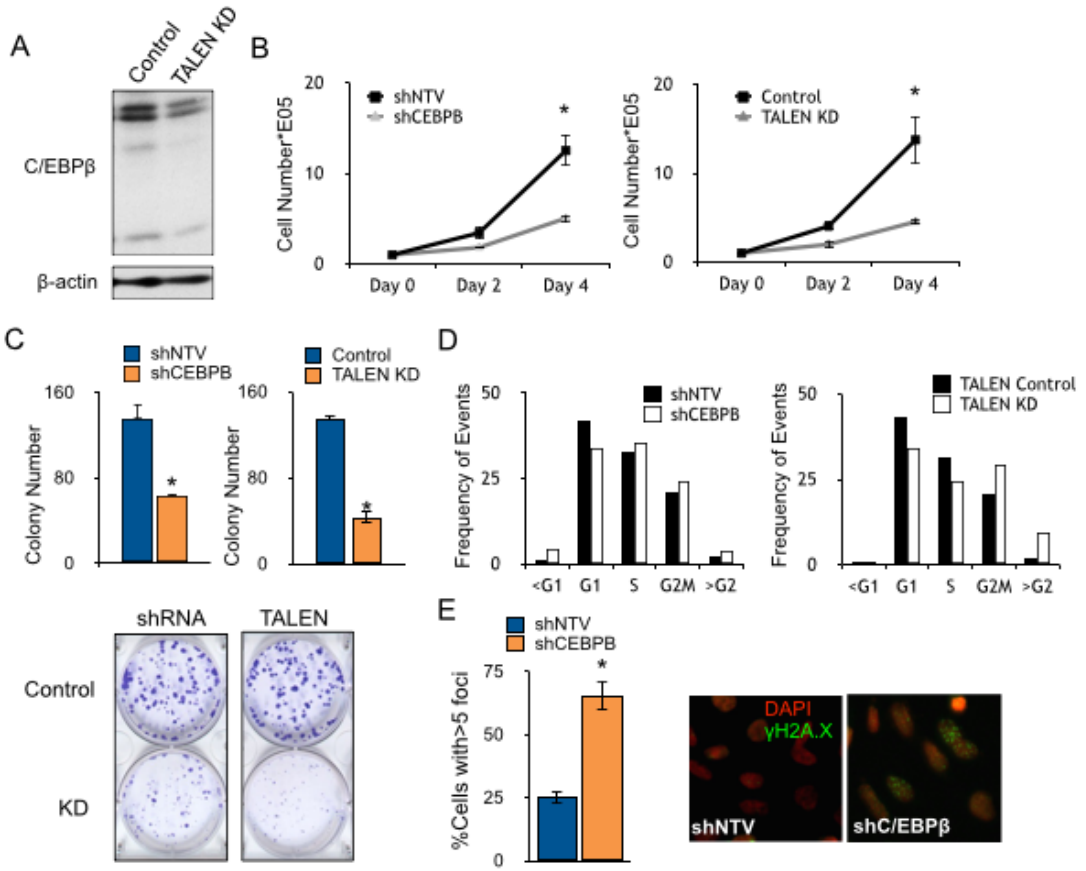


**Figure 6. C/EBPβ promotes PC3 growth and suppresses DNA damage. (A)** Western blot analysis of C/EBPβ expression in PC3 cells with edited CEBPB by TALEN. **(B)** Quantification of PC3 cell growth after 3 and 5 days of culture. **(B)** Quantification of clonogenic growth in PC3 cells. **(C)** Determination of cell cycle fractions in PC3 cultures by FACS analysis of DNA content by PI staining. **(D)** Quantification of DNA double strand breaks in PC3 cultures by immunofluorescent staining of γH2A.X. Bar graphs represent average of three experiments. Error bars: SEM. *p<0.01.

Lastly, we evaluated the role of C/EBPβ in prostate tumor growth and sensitivity to bortezomib. We subcutaneously engrafted NSG mice with shNTV or shCEBPB PC3 cells in matrigel and when tumor reached sizes between 100 and 300mm$^3$ mice were placed on a doxycycline-laced animal feed for two days and then received IP injection of bortezomib (1mg/kg) or vehicle (DMSO 1: 5000 in saline) on days 1, 4 and 8. Tumor volume was evaluated by caliper measurement. We found that suppression of C/EBPβ significantly reduced the growth rate of prostate tumors (Fig. 8A). Significantly, we also found that administration of bortezomib did not significantly affect the growth rate of control shNTV tumors and that suppression of C/EBPβ sensitized these tumors to bortezomib treatment. These results demonstrate that C/EBPβ promotes the growth of castrate-resistant prostate cancer and resistance to bortezomib *in vivo*.

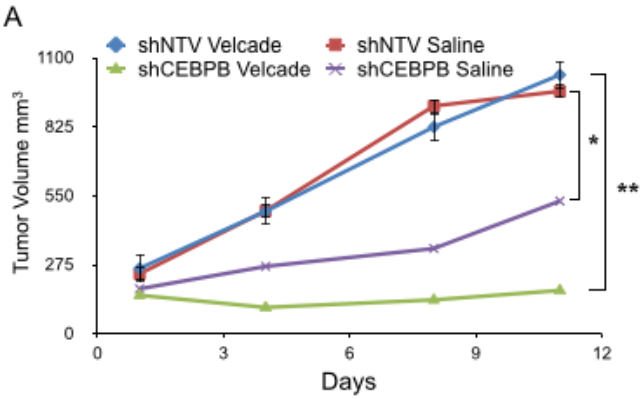


**Figure 7. C/EBPβ promotes prostate tumor growth and resistance to bortezomib *in vivo*. (A)** Quantification of PC3 tumor growth by caliper measurement in NOG mice treated with vehicle or bortezomib (1mg/kg) on days 1, 4 and 8. *p<0.01; *p<0.001

*Methods*
The methods used for these experiments have been described in the attached publication. Barakat et al., 2015. Statistically significant differences in tumor growth rates were analyzed by linear regression analysis using Graph Pad Prism statistical software.

*Discussion of goals not met:*
The findings of these studies reveal that C/EBPβ translational isoforms can be either beneficial or detrimental to prostate cancer cell survival depending on the treatment type. Our studies in androgen sensitive prostate cancer lines show that these cells preferentially express the LIP isoform and that the isoform ratio is maintained when C/EBPβ protein levels are increased during androgen deprivation. Our findings suggest that the higher levels of LIP are critical for maintaining mTOR activation because when we increase LAP expression, mTOR activity decreases. Further, C/EBPβ expression increases in castrate-resistant prostate cancer and suppression of C/EBPβ decreased castrate-resistant prostate cancer tumor growth. Conversely, LAP expression appeared to be protective when cells were challenged with the proteasome inhibitor, bortezomib. Because bortezomib causes proteotoxicity by accumulation of mis-folded proteins, suppression of protein synthesis by decreasing mTOR activity could promote cell survival in this context. These studies suggest that modulating mTOR activity is a key function of C/EBPβ for adaptation to metabolic stress in prostate cancer cells.

Contrary to our expected results, we have found that inhibition of mTOR leads to down-regulation of *CEBPB* transcripts and suppression of all three isoforms. However, this finding does not change the overall goals or objectives of the project because the purpose was to determine the critical regulators of C/EBP translational isoforms in prostate cancer cells and whether the change in isoform ratio could promote cell survival or therapeutic resistance. We found that cap-dependent translation was a critical regulator of LAP isoform expression in prostate cancer cell lines. We had intended to generate stable prostate cancer cell lines with ectopic expression of LAP or LIP isoforms. This proved challenging because C/EBPβ promotes cellular senescence when over-expressed in LNCaP prostate cancer cells, and appeared to generate an epithelial phenotype in PC3 cells (not shown). We are cloning these constructs into the piggybac tet-on vector for inducible over-expression of these isoforms in prostate cancer cells. For the next reporting period, we will focus our efforts on the role of C/EBPβ isoforms and inhibitors of cap-dependent translation in regulating survival during during androgen deprivation *in vivo* and *in vitro*. These experimental efforts will address the goals of major tasks 2 and 3.

4. Other achievements:
Nothing to report.

- **What opportunities for training and professional development has the project provided?**
  There were several opportunities for training and professional development during the award period that Dr. Barakat took advantage of to achieve the planned training goals. Dr. Barakat presented his findings at the 2015 American Association for Cancer Research meeting in Philadelphia, PA and at two internal Johns Hopkins research events: Prostate Cancer Research Day and Fellows Research day. These events provided Dr. Barakat with

the opportunity to network with other professionals with similar research interests and served as a means to improve upon his scientific communication skills. Dr. Barakat had one-on-one meetings with his mentors, Dr. Alan Friedman and Dr. Samuel Denmeade, on several occasions where he was able to present his findings and receive feedback on the progress and direction of his ongoing studies. Dr. Barakat also had the opportunity to improve upon his grantsmanship skills by writing a K99/R00 pathway to independence award with the help of his mentor, Dr. Alan Friedman. Under the close guidance of Dr. Friedman, the grant application was successfully completed during the June submission cycle and a scientific review meeting has been set for October of this year. The original plan was to submit this grant during the second year of the award, but because of changes in eligibility criteria, the grant had to be submitted earlier. This experience was not only a grantsmanship training opportunity, but also helped Dr. Barakat to improve his skills in planning and developing a research project with long-term goals and represents initial steps towards developing an independent scientific career.

Dr. Barakat also improved upon several laboratory skills during this award period including *in vivo* animal studies, subcutaneous prostate tumor xenografting, surgical castration, evaluating tumor volume by caliper measurement and administration of drugs by IP injection in mice. Training in these skills were overseen by Marc Rosen, a research associate in Dr. Sam Denmeade's laboratory with over ten years of experience in mouse prostate cancer models.

- **How were the results disseminated to communities of interest?**

  Nothing to report.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  During the next reporting period, we will focus our efforts on determining whether increasing the LAP:LIP C/EBPβ isoform ratio by pharmacological blockade of cap-dependent translation can promote cell death in combination with anti-androgens *in vitro* or delay castrate-resistant growth in animal models of prostate cancer *in vivo*. To accomplish the research goals of this award we will carryout the following experiments:

  *1) Determine the role of the C/EBPβ isoform ratio in androgen-sensitive prostate cancer cell sensitivity to anti-androgens.* We will transduce LNCaP cells with piggybac tet-on vectors (described in Barakat et al., 2015) for inducible expression of LAP and LIP isoforms. We will then utilize these cell lines to determine the role of individual isoforms in sensitivity to anti-androgens or blockade of cap-dependent translation. We will evaluate LNCaP cell death *in vitro* by WST-1 assay and flow cytometry analysis of annexin V staining. Further, we will test whether C/EBPβ promotes survival during combined treatment of anti-androgens and 4E/Gi using cells with shRNA knockdown of C/EBPβ.

  *2) Evaluate the role of C/EBPβ isoforms in regulation of mTOR activity.* We found that blockade of cap-dependent translation increased LAP and REDD1 expression and suppressed mTOR activity in LNCaP cells. We will evaluate whether the increase in LAP was critical for suppression of mTOR activation under these conditions utilizing LNCaP cells expressing shRNA targeting C/EBPβ. Under similar conditions, we will knockdown the expression of REDD1 with siRNA to evaluate whether REDD1 is a critical target of C/EBPβ which control mTOR activity in prostate cancer cells. Further, we will evaluate mTOR activity and REDD1 expression in LNCaP cells ectopically expressing LAP or LIP isoforms. We expect that LAP will suppress mTOR activation by REDD1 in LNCaP cells.

  *3) Evaluate the role of C/EBPβ translational isoforms in regulating progression to castrate-resistant prostate cancer in vivo.* We will transplant NOG mice with LNCaP cells expressing shRNA targeting C/EBPβ or control shRNA and following development of solid tumors, we will surgically castrate animals and treat them with 4E1RCAT (15mg/kg, i.p. daily for two weeks), a recently developed chemical inhibitor of cap-dependent translation that is suitable for *in vivo* use [21]. We will evaluate tumor growth for 8 weeks after castration by caliper measurement. In a separate group of mice, we will evaluate expression of C/EBPβ isoforms, cleaved caspase-3 and activation of mTOR by phosphorylation of p70S6K by Western blot analysis 72 hours after castration. We will further explore the role of bortezomib in regulating cap-dependent translation in prostate cancer cells and publish our findings on the role of C/

EBPβ in resistance to bortezomib and the role of C/EBPβ isoforms in promoting progression to castrate-resistant disease as two separate studies.

**4. IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

  The findings of this research project further our knowledge of the mechanisms that govern protein metabolism in prostate cancer cells and how manipulating C/EBPβ can affect prostate cancer response to androgen deprivation or chemotherapy. The protein kinase known as mTOR is a key driver of protein synthesis in mammalian cells and is hyper-activated in 40-70% of advanced prostate cancers. Because cancer cells grow at much faster rates than ordinary cells, activation of mTOR is necessary to keep up with metabolic demands of the growing tumor. Androgen deprivation therapy, the primary treatment for advanced prostate cancer, was recently suggested to increase mTOR activity and that this event could eventually drive resistance [1]. Our research group recently discovered that treating prostate cancer cells with androgen deprivation therapy increased the levels of another protein called CCAAT/Enhancer binding protein beta (C/EBPβ), which has been reported to regulate mTOR activity. Cells make short and long isoforms of this protein, which can promote cell proliferation or growth arrest and survival, respectively. We discovered that the long C/EBPβ isoform suppressed the activity of mTOR in prostate cancer cells and that we could use different drugs to promote the expression of the long isoform. Increasing expression of the long isoform in combination with androgen deprivation further suppressed mTOR activity. If we blocked expression of C/EBPβ in our prostate cancer cells, we found that the development of castrate-resistant tumor growth was delayed in mice transplanted with these cells. The results of this research project increased our understanding of how the isoforms of C/EBPβ are regulated, how these isoforms could regulate protein synthesis and cellular growth in prostate cancer cells and how altering the levels of individual C/EBPβ isoforms could effect resistance to chemotherapy or anti-androgens.

- **What was the impact on other disciplines?**

  The findings of this research project increase our basic understand of the mechanisms that govern the control of C/EBP translational isoforms and how the individual isoforms affect cellular protein metabolism. C/EBPβ is a critical player in cell metabolism and physiology. It is critical for promoting white adipocyte differentiation and it is expressed in the skeletal muscle and liver where it has been shown to play a role in regulating insulin sensitivity and glucose production and autophagy, respectively [28-30]. The findings of our studies are in line with the crucial metabolic role of C/EBPβ and show that the regulation of C/EBPβ isoforms can suppress or promote protein synthesis.

- **What was the impact on technology transfer?**

  Nothing to report.

- **What was the impact on society beyond science and technology?**

  Nothing to report.

**5 CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  Nothing to report.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  Nothing to report.
- **Changes that had a significant impact on expenditures**
  Nothing to report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  Nothing to report.
- **Significant changes in use or care of human subjects**
  Nothing to report.
- **Significant changes in use or care of vertebrate animals.**
  Nothing to report.
- **Significant changes in use of biohazards and/or select agents**
  Nothing to report.

## 6 PRODUCTS:

- **Publications, conference papers, and presentations**
  - **Journal publications.**
    Barakat DJ, Zhang J, Barberi T, Denmeade SR, Friedman AD, Paz-Priel I. CCAAT/ Enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells. Oncogene. doi: 10.1038/onc.2015.41 [Epub, ahead of print]; 2015. Published. Acknowledgment of federal support: yes.
  - **Books or other non-periodical, one-time publications.**
    Nothing to report.
  - **Other publications, conference papers, and presentations.**
    CCAAT/enhancer binding protein beta facilitates castrate-resistant prostate cancer cell growth and sensitivity to mTOR inhibitors. David J. Barakat, Jing Zhang, Alan D. Friedman, Samuel R. Denmeade, Ido Paz-Priel. Johns Hopkins University, Baltimore, MD. American Association for Cancer Research Annual Meeting, Philadelphia, PA, April 19th, 2015.

- **Website(s) or other Internet site(s)**
  Nothing to report.
- **Technologies or techniques**
  Nothing to report.
- **Inventions, patent applications, and/or licenses**
  Nothing to report.
- **Other Products**
  Nothing to report.

## 7 PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
  - Name:
    *David Barakat*
  - Project Role:
    *Principle Investigator*
  - Researcher Identifier (e.g. ORCID ID):
    *ORCID ID: 0000-0001-7057-178X*
  - Nearest person month worked:
    *12*
  - Contribution to Project:
    *Dr. Barakat performed all of the experiments proposed for this award, analyzed and interpreted results and presented data at conferences.*
  - Funding Support:
    *PC131609 DOD Postdoctoral Fellowship Award Barakat (PI) 7/01/2014-6/30/2016*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Dr. Alan Friedman's (primary mentor) active R01 grant: Regulation of Monocyte versus Granulocyte Lineage Specification has closed. Subsequent to this, he has been awarded an Alex's Lemonade Stand Foundation grant and a Hyundai Hope on Wheels grant (see below).

  28520 (Friedman) 7/01/14-6/30/16 1.8 Cal
  Alex's Lemonade Stand Foundation $125,000
  Converting the Glioblastoma Multiforme Tumor-Associated Macrophage Phenotype
  The aims of this this project are to determine whether intra-cranial GL261 glioma tumor growth is reduced in mice with reduced or absent C/EBPβ or NF-κB p50, associated with M2 to M1 tumor-associated and marrow macrophage conversion; to determine whether C/EBPβ variants that bind NF-κB p50 but not DNA rescue the M2 marrow macrophage phenotype; and to determine whether targeting PD-1 synergizes with lack of C/EBPβ or NF-κB p50 to slow GL261 glioma tumor growth via reduced T cell blockade.

45945MD (Friedman) 12/01/15-11/30/18 0.6 Cal
Hyundai Hope on Wheels $80,000
Targeting M-CSF to PU.1 Signaling To Convert the GBM Tumor-Associated Macrophage Phenotype
The aims of this project are to determine whether intra-cranial GL261 glioma tumor growth is reduced in mice with reduced PU.1, associated with reduced M2 tumor-associated macrophages and increased anti-tumor T cell immunity, to determine whether inhibition of MCSFR tyrosine kinase activity synergizes with reduced PU.1 to favor M1 over M2 TAM polarization and to slow/prevent GBM tumor growth, and to identify the MCSFR signaling pathway most critical for PU.1 activation or induction and determine whether inhibition of this pathway synergizes with MCSFR inhibition to favor M1 TAM polarization.

- **What other organizations were involved as partners?**
  Nothing to report.

## 8 SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
  Nothing to report.
- **QUAD CHARTS:**
  Nothing to report.

## 9 APPENDICES:

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**Publications and meeting abstracts:**

*CCAAT/Enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells.* Barakat DJ, Zhang J, Barberi T, Denmeade SR, Friedman AD, Paz-Priel I. Oncogene. 2015 Mar 16. doi: 10.1038/onc.2015.41. [Epub ahead of print]

CCAAT/enhancer binding protein beta facilitates castrate-resistant prostate cancer cell growth and sensitivity to mTOR inhibitors. David J. Barakat, Jing Zhang, Alan D. Friedman, Samuel R. Denmeade, Ido Paz-Priel. Johns Hopkins University, Baltimore, MD. American Association of Cancer Research Annual Meeting, April 19th, 2015.

Oncogene (2015), 1–11

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ORIGINAL ARTICLE

# CCAAT/Enhancer binding protein β controls androgen-deprivation-induced senescence in prostate cancer cells

DJ Barakat[1], J Zhang[1], T Barberi[1], SR Denmeade[2], AD Friedman[1] and I Paz-Priel[1]

The processes associated with transition to castration-resistant prostate cancer (PC) growth are not well understood. Cellular senescence is a stable cell cycle arrest that occurs in response to sublethal stress. It is often overcome in malignant transformation to confer a survival advantage. CCAAT/Enhancer Binding Protein (C/EBP) β function is frequently deregulated in human malignancies and interestingly, androgen-sensitive PC cells express primarily the liver-enriched inhibitory protein isoform. We found that C/EBPβ expression is negatively regulated by androgen receptor (AR) activity and that treatment of androgen-sensitive cell lines with anti-androgens increases C/EBPβ mRNA and protein levels. Accordingly, we also find that C/EBPβ levels are significantly elevated in primary PC samples from castration-resistant compared with therapy-naive patients. Chromatin immunoprecipitation demonstrated enhanced binding of the AR to the proximal promoter of the *CEBPB* gene in the presence of dihydroxytestosterone. Upon androgen deprivation, induction of C/EBPβ is facilitated by active transcription as evident by increased histone 3 acetylation at the C/EBPβ promoter. Also, the androgen agonist R1881 suppresses the activity of a *CEBPB* promoter reporter. Loss of C/EBPβ expression prevents growth arrest following androgen deprivation or anti-androgen challenge. Accordingly, suppression of C/EBPβ under low androgen conditions results in reduced expression of senescence-associated secretory genes, significantly decreased number of cells displaying heterochromatin foci and increased numbers of Ki67-positive cells. Ectopic expression of C/EBPβ caused pronounced morphological changes, reduced PC cell growth and increased the number of senescent LNCaP cells. Lastly, we found that senescence contributes to PC cell survival under androgen deprivation, and C/EBPβ-deficient cells were significantly more susceptible to killing by cytotoxic chemotherapy following androgen deprivation. Our data demonstrate that upregulation of C/EBPβ is critical for complete maintenance of androgen deprivation-induced senescence and that targeting C/EBPβ expression may synergize with anti-androgen or chemotherapy in eradicating PC.

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## INTRODUCTION

Prostate cancer (PC) is the most prevalent malignancy in adult men in the United States.[1] Although early detection and treatment of localized disease is often curative, PC remains a leading cause of cancer death. Anti-androgen therapy is the most effective approach in patients with advanced disease and induces significant responses in almost all patients.[2] However, androgen deprivation achieved by pharmacologic or surgical castration results in only limited apoptosis of tumor cells[3,4] and accordingly only partial tumor regression. Indeed, after a period of disease control, most patients develop castration-resistant growth and PC progression, which is responsible for the majority of the morbidity and mortality associated with this disease.[2] Identifying mechanisms that engender castration resistance is crucial for the design of future therapeutic strategies. Progress has been made understanding the mechanisms associated with eventual emergence of castration-resistant PC (CRPC). However, less is known about the early adaptation associated with androgen deprivation.[5–7]

Members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors are characterized by a conserved C-terminus, which contains both a DNA-binding basic region and leucine-zipper, collectively referred to as the bZIP domain. C/EBPβ is a widely expressed transcription factor that promotes proliferation or terminal differentiation and growth arrest in several different cell types.[8] These opposing functions seem to be regulated by the expression of different C/EBPβ translational isoforms from three in-frame start codons within an intron-less mRNA.[9,10] The two high molecular weight C/EBPβ isoforms, termed liver-enriched activating proteins (LAP and LAP*), contain N-terminal transactivation domains, whereas the liver-enriched inhibitory protein lacks these transactivation domains. Liver-enriched inhibitory protein can dominantly inhibit LAPs and other C/EBP members via heterodimerization or by recruiting transcriptional repressors.[11] C/EBPβ activity affects several facets of PC disease progression. C/EBPβ regulates the expression of steroidogenic genes including StAR and cytochrome p450 aromatase,[12,13] and its activity is modulated in response to dihydrotestosterone, estrogen and progesterone.[14–17] It has also been suggested that C/EBPβ can act as a co-repressor of the androgen receptor (AR) in PC.[18,19] Although C/EBPβ is not detected in healthy prostate, luminal epithelial cells upregulate C/EBPβ in the case of proliferative-inflammatory atrophy, a precursor to PC, [20] and C/EBPβ participates in the regulation of metastatic genes and PC cell survival.[21,22] However, the contribution of C/EBPβ to the emergence of castration-resistant growth has not been previously investigated.

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Cellular senescence is a stable cell cycle arrest that occurs in response to a variety of intrinsic and extrinsic sublethal stress stimuli.[23,24] Accumulating data point to an important role of senescence in cancer progression.[23,24] Recently, several groups demonstrated that in response to androgen deprivation, PC cells undergo senescence, and that the acquisition of senescence is associated with emergence of castrate-resistant growth.[5–7] In other lineages, C/EBPβ and its downstream target genes are critical for the induction and maintenance of oncogene-induced senescence, associated with overexpression of activated Ras or BRAF.[8,25,26] C/EBPβ can directly bind to target gene promoters and enhancers to induce senescence-associated factors IL-6 and IL-8, but can also suppress E2F-1 target genes and induce growth arrest dependent on E2F:pRb.[8]

We now demonstrate that upon androgen deprivation, C/EBPβ is rapidly upregulated in androgen-sensitive PC cells and that AR binds to and suppresses the C/EBPβ proximal promoter. Increased expression of C/EBPβ under these conditions is necessary for acquisition of the senescent phenotype. Accordingly, preventing C/EBPβ upregulation increases the susceptibility of PC cells to apoptosis induced by chemotherapy.

## RESULTS

### CRPC is associated with increased *CEBPB*

To determine whether C/EBPβ levels correlate with human PC progression, we interrogated the Oncomine database.[27] In the Grasso *et al*.[28] data set that included gene expression patterns from 28 benign prostate tissues, 59 localized PC and 35 CRPC samples, *CEBPB* expression was significantly ($P < 1.9 \times 10^{-6}$) elevated in CRPC compared with localized disease (Figures 1a and b).

### Inhibition of AR induces *CEBPB* transcription

Treatment of LNCaP cells with the synthetic AR agonist R1881 for 24 h results in a dose-dependent 2.5-fold decrease in *CEBPB* mRNA and protein expression (Figures 2a and b), and as expected, prostate-specific antigen transcript levels increased under these conditions. Conversely, culturing LNCaP cells in androgen-depleted media (ADM) for 7 days resulted in a significant 3.8-fold increase in C/EBPβ expression (Figure 2c). Pharmacologic inhibition of the AR using bicalutamide resulted in a dose-dependent rise in *CEBPB* transcript abundance, achieving a 7.5-fold increase at the highest dose tested (Figure 2d). Accordingly, we detected increased protein levels of C/EBPβ in both LNCaP and LAPC4 cells treated with bicalutamide or flutamide (Figure 2e). As bicalutamide or flutamide may have an AR agonist effect, we also tested the effect of enzalutamide, which does not have agonistic effects. Similar to bicalutamide, incubation with 20 μM enzalutamide resulted in increased C/EBPβ levels (Figure 2f). *CEBPB* RNA levels were rapidly upregulated within 4 h of exposure of LNCaP cells to bicalutamide (Figure 2g).

To assess binding of AR to the *CEBPB* promoter, LNCaP cells were cultured in full media and subjected to chromatin immunoprecipitation analysis. Precipitated DNA was amplified using primers spanning the proximal (−131 to − 242 bp) or distal (−2098 to − 1983 bp) regions of the human *CEBPB* promoter. We observed AR binding to the proximal but not the distal region (Figure 3a). Next, CEBPB-luc, containing proximal promoter region (−888 to +64) linked to a luciferase reporter, was co-transfected into LNCaP or DU145 PC cells with CMV-β-galactosidase as internal control. Reproducibly, luciferase activity significantly decreased by 2.5-fold in LNCaP cells cultured with 1 nM R1881 for 24 h compared with vehicle control (Figure 3b). This effect on *CEBPB* promoter activation was mediated by the AR as R1881 did not reduce luciferase activity in similarly transfected DU145 cells which lack AR. Treatment of LNCaP cells with bicalutamide for 4 h induced acetylation of histone H3 bound to the *CEBPB* proximal promoter, whereas culture with dihydrotestosterone suppressed this mark of active transcription (Figure 3c). Importantly, we did not observe significant changes in the half-life of *CEBPB* RNA in response to bicalutamide, indicating that the stability of *CEBPB* transcripts was unaffected (Figure 3d). Collectively, these results show that the AR suppresses transcription of *CEBPB*.

### Ectopic expression of C/EBPβ suppresses LNCaP cell growth

Because C/EBPβ expression was inversely regulated by AR activity, an essential signal for PC cell growth, we next evaluated whether ectopic expression induces growth arrest similar to androgen deprivation. C/EBPβ was inducibly expressed in LNCaP cells using the Tet-regulated transposon-based piggyBac vector[29] and cells with stable integration of the transgene or an empty vector (PB-TRE) control were selected by puromycin (2 mg/ml). Treatment of LNCaP PB-CEBPB cells with doxycycline for 5 days induced an increase in the expression of C/EBPβ 2.5-fold (Figure 4a), similar to the increase seen in LNCaP cells cultured in enzalutamide. C/EBPβ forced expression was associated with increased levels of the cell cycle inhibitors $p16^{INK4A}$ and $p15^{INK4B}$ and a flattened morphology (Figures 4a and b). Next, we tested the effect of C/EBPβ overexpression on LNCaP cell proliferation. Equal numbers of LNCaP PB-CEBPB or PB-TRE control cells were seeded in doxycycline-containing media and enumerated after 3 and 5 days. Ectopic C/EBPβ expression resulted in a significant 2.3-fold decreased rate of proliferation (Figure 4c) and a significantly increased number of Ki67-negative cells 5 days after doxycycline treatment (Figure 4d) without increased cell death (Figure 4e). These data demonstrate that elevated levels of C/EBPβ are sufficient to suppress the growth of androgen-sensitive PC cells.

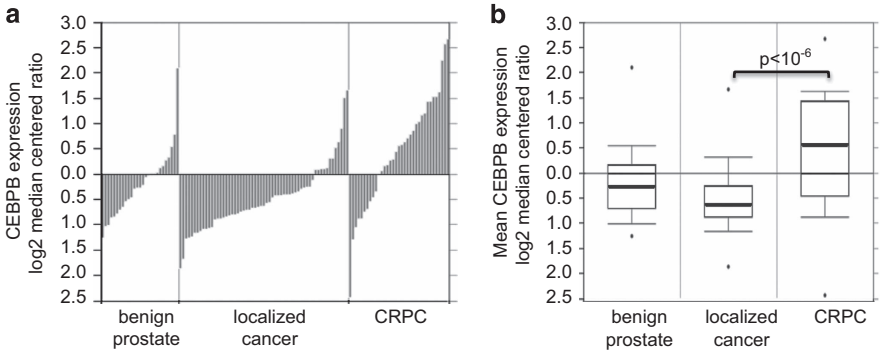


**Figure 1.** C/EBPβ expression increases in CRPC. Individual patient (**a**) and mean (**b**) *CEBPB* expression as log2 median centered ratio for benign prostate, localized PC and CRPC.

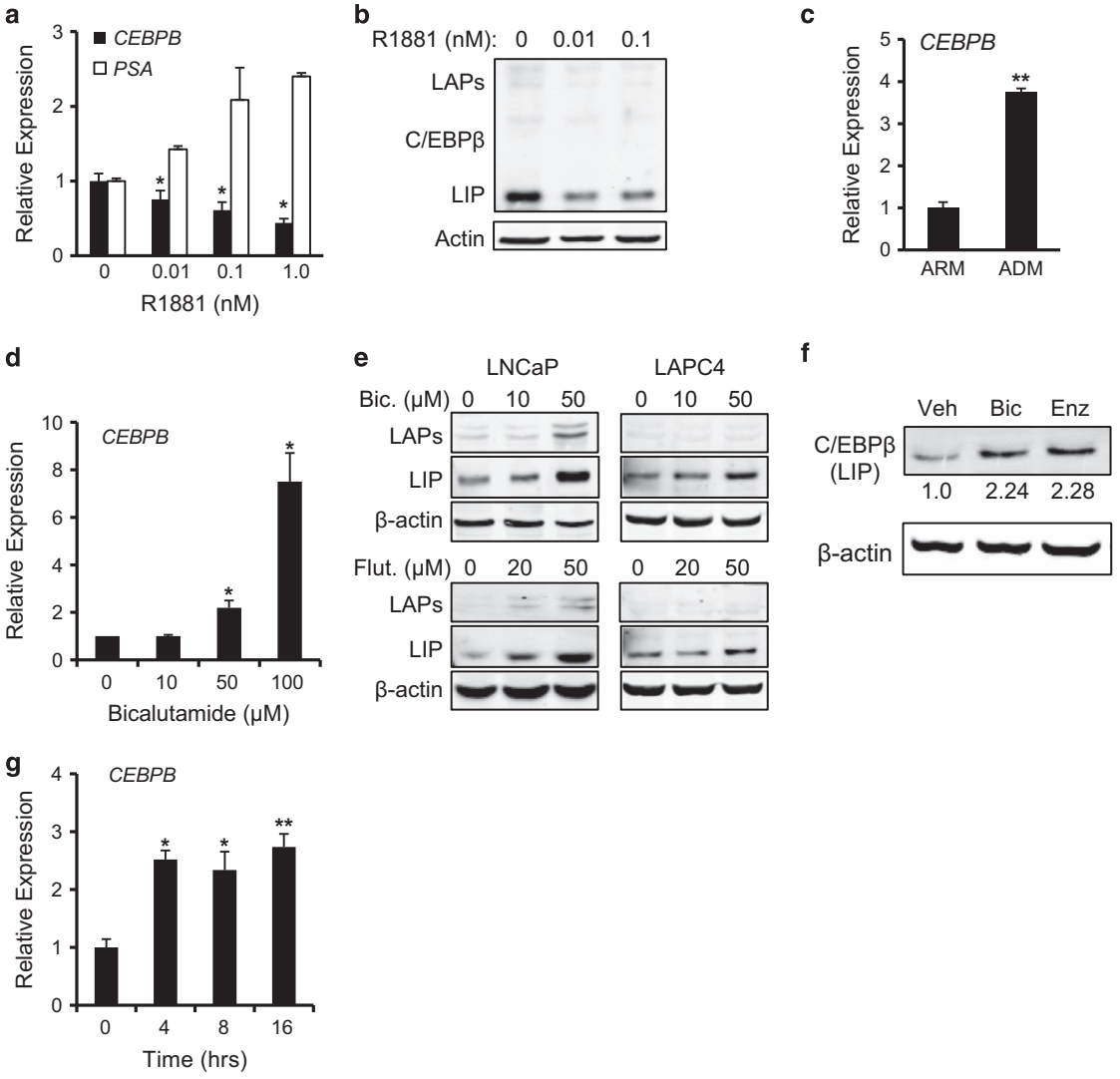


**Figure 2.** C/EBPβ expression is regulated by AR activity in PC cell lines. LNCaP cells were cultured in the indicated concentrations of R1881 for 24 h and RNA (**a**) or protein (**b**) were analyzed for the expression of the indicated gene products. (**c**) LNCaP cells were cultured in ARM or ADM for 9 days and *CEBPB* RNA levels analyzed. Mean and s.d. from three independent experiments are shown. (**d**) *CEBPB* transcripts levels in LNCaP cells cultured with the indicated concentration of bicalutamide were measured in three independent experiments using qRT–PCR. (**e**) LNCaP or LAPC4 cells were cultured with the indicated doses of bicalutamide (Bic), flutamide (Flut) or (**f**) enzalutamide (enz) for 24 h, and the cell lysates were subjected to western blotting. Representative gels with relative band intensity values are shown. (**g**) LNCaP cells were exposed to bicalutamide at 50 μM and C/EBPβ expression was assessed at the indicated time points. The average normalized *CEBPB* transcripts levels from three independent experiments are shown. $*P < 0.01$, $**P < 0.001$.

## C/EBPβ is required for complete maintenance of androgen-deprivation-induced growth arrest

Persistence of PC cells under androgen-deprived conditions is an initial step towards development of castration-resistant growth. Upon androgen deprivation, LNCaP cells undergo cell cycle arrest and enter a senescent state.[5–7] Importantly, androgen deprivation-induced senescence is only partly reversible and cells continue to display senescence markers and poor proliferation after re-exposure to androgen.[6] Because C/EBPβ upregulation was associated with androgen deprivation and its expression sufficient to suppress LNCaP proliferation, we next targeted C/EBPβ to test its function in androgen-deprivation-induced growth arrest. We utilized two independent methods: inducible shRNA and transcription activator-like effector nucleases (TALENs).[30,31] We designed a pair of TALENs to target the *CEBPB* gene, transfected LNCaP cells, and screened individual clones for C/EBPβ expression. Subclone 6, was identified as having *CEBPB* knockdown (presumably due to incomplete targeting of all alleles in the polyploid LNCaP cells) (Figure 5a), and used in our subsequent experiments. A complete stable deletion of all *CEBPB* alleles could not be achieved, suggesting C/EBPβ plays a critical role for cell survival. As control we employed subclone 1 in which C/EBPβ expression is similar to that observed in parental cells. In addition, C/EBPβ expression was effectively knocked down in LNCaP cells utilizing a doxycycline-inducible shRNA against *CEBPB* (shCEBPB) compared with a non-targeting vector control (shNTV) (Figure 5b). We used flow cytometry to investigate the effect of C/EBPβ depletion on PC cell cycle distribution (Figure 5c). For these studies, we employed cells expressing the inducible shRNA as stable deletion of C/EBPβ via TALEN required adaptation to low C/EBPβ levels. Cells expressing shCEBPB or shNTV both went into G1 cell cycle arrest when cultured in ADM (Figure 5c).

The growth arrest induced by androgen deprivation is associated with changes in cell cycle inhibitors including $p16^{INK4A}$, $p15^{INK4B}$ and $p21^{WAF1}$. Because C/EBPβ has been shown to regulate the expression of these genes, we next evaluated their

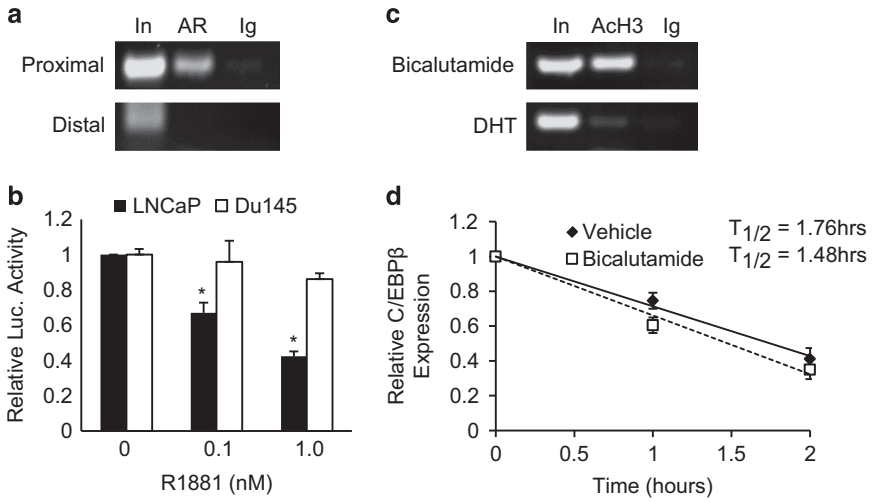


**Figure 3.** AR binds and represses the CEBPB gene. (**a**) LNCaP cells were subjected to chromatin immunoprecipitation analysis with antibodies against AR or normal rabbit IgG (Ig). PCR was used to amplify DNA fragments centered at − 187 bp (proximal) or an upstream fragment centered at − 2040 bp (distal) of the *CEBPB* gene. (**b**) LNCaP or DU145 cells were transiently transfected with CEBPB-Luc and cultured with vehicle or R1881 at the indicated dose. Fold activation of the reporter relative to vehicle-treated cells was determined after normalization to β-galactosidase activity. The average from three independent experiments is presented. (**c**) LNCaP cells were cultured in the presence of bicalutamide (50 μM) or dihydroxytestosterone (DHT) (10 nM) for 4 h and subjected to chromatin immunoprecipitation using antibodies against acetylated histone 3 (AcH3) or normal rabbit IgG (Ig). A representative gel of PCR-amplified proximal promoter DNA fragment is shown. (**d**) LNCaP cells were cultured in the presence of vehicle or bicalutamide (50 μM) and after 4 h actinomycin D (5 μg/ml) was added to the media. Total cellular RNA was harvested at the indicated time points, reverse-transcribed to cDNA and relative *CEBPB* transcript levels were measured using qRT–PCR. The slope of a linear best-fit line for each group was determined to calculate the *CEBPB* RNA half-life, based on three repetitions.

expression in LNCaP cells following androgen deprivation. The levels of $p16^{INK4A}$, $p15^{INK4B}$ and $p21^{WAF1}$ proteins were diminished in C/EBPβ-deficient cells compared with shNTV cultured in androgen-replete media (ARM) or ADM (Figure 5d). In contrast to $p15^{INK4B}$ and $p21^{WAF1}$, $p16^{INK4A}$ protein levels mildly increased in shCEBPB cells following culture in ADM, compared with culture in ARM. However, $p16^{INK4A}$ levels in shCEBPB cells are markedly lower compared with shNTV control cells cultured in either ARM or ADM (Figure 5d).

To evaluate whether C/EBPβ is required to maintain growth arrest of LNCaP cells challenged by androgen deprivation, we pre-cultured LNCaP cells harboring shNTV or shC/EBPβ in ADM or ARM with doxycycline for 7 days, re-seeded equal number of cells in ARM and enumerated viable cells 5 days later (Figure 6a). As expected from the observed inhibition of G1 to S cell cycle progression (Figure 5c), culture in ADM for 7 days resulted in diminished cell accumulation (not shown). Although in ARM, the rate of proliferation of shCEBPB cells was modestly lower than shNTV cells, C/EBPβ knockdown was associated with a better, although incomplete, recovery of proliferation after pre-culture in ADM (Figure 6b).

Charcoal-stripped fetal bovine serum (FBS) lacks androgen and multiple other growth factors. To define the specific effect of androgen depletion, LNCaP cells harboring shNTV or shCEBPB were pre-cultured with doxycycline and bicalutamide, enzalutimide or vehicle for 4 days, and subsequently re-plated at equal numbers in ARM without inhibitors (Figure 6a). In contrast to shNTV cells, which demonstrated twofold decreased recovery, *CEBPB*-depleted cells completely recovered their proliferation once released from AR inhibition (Figure 6c). To better reflect recovery and adjust for the different proliferation rate in ARM of shCEBP compared with shNTV cells, we also plotted these data as a ratio of growth of cells pre-cultured in ADM, bicalutamide or enzalutimide to cells pre-cultured in ARM (Figure 6d). This normalization highlights the recovery of proliferation of shCEBPB compared with shNTV cells in each experiment, indicating that C/EBPβ is required for maintenance of complete growth arrest induced by androgen deprivation and its suppression alleviates the phenotype, at least in part.

### C/EBPβ elevation induces senescence

Because C/EBPβ seemed to play a role in maintaining growth arrest following androgen deprivation, we next evaluated whether overexpression of C/EBPβ was sufficient to induce senescence in LNCaP cells. Senescent cells are characterized by an increase in cell volume, granularity and lysosomal mass indicated by senescence-associated β-galactosidase (SA-β-gal) activity.[32] Compared with control PB-TRE, PB-CEBPB cells had a significant increase in the number SA-β-gal-positive cells and the level of cell granularity, as assessed by side scatter (Figures 7a and b). Expression of several secreted gene products is elevated in senescent cells and is referred to as the senescence-associated secretory phenotype.[24] Release of these secreted factors promotes paracrine growth arrest, and C/EBPβ was shown to be central to induction of senescence-associated secretory phenotype genes.[25,33,34] Accordingly, overexpression of C/EBPβ led to a significant increase in the transcript levels of two senescence-associated secretory phenotype-associated genes, *IL8* and *IGFBP3* (Figure 7c). Similarly, we observed increased IGFBP3 and IL8 levels upon androgen deprivation, which was abrogated by either *CEBPB* shRNA or TALEN targeting (Figures 7d and e). Another feature of senescent cells is accumulation of tightly packed heterochromatin foci characterized by increased di- or trimethylated histone 3 on lysine 9 (H3K9me2, H3K9me3).[35] When cultured in ADM, LNCaP-shNTV cells display a twofold increase in the number of heterochromatin foci-positive cells. In contrast, we did not observe an increase in heterochromatin foci in shCEBPB cells cultured under similar conditions (Figure 7f). Senescent cells exit the cell cycle, and stain negative for Ki67. A similar proportion of shNTV or shCEBPB cells expressed Ki67 in ARM. However, the proportion of Ki67-negative cells after 1 week of culture in ADM was twofold lower in cells lacking C/EBPβ (Figure 7g). Together, these data indicate that in PC cells C/EBPβ is necessary for induction of senescence upon androgen deprivation.

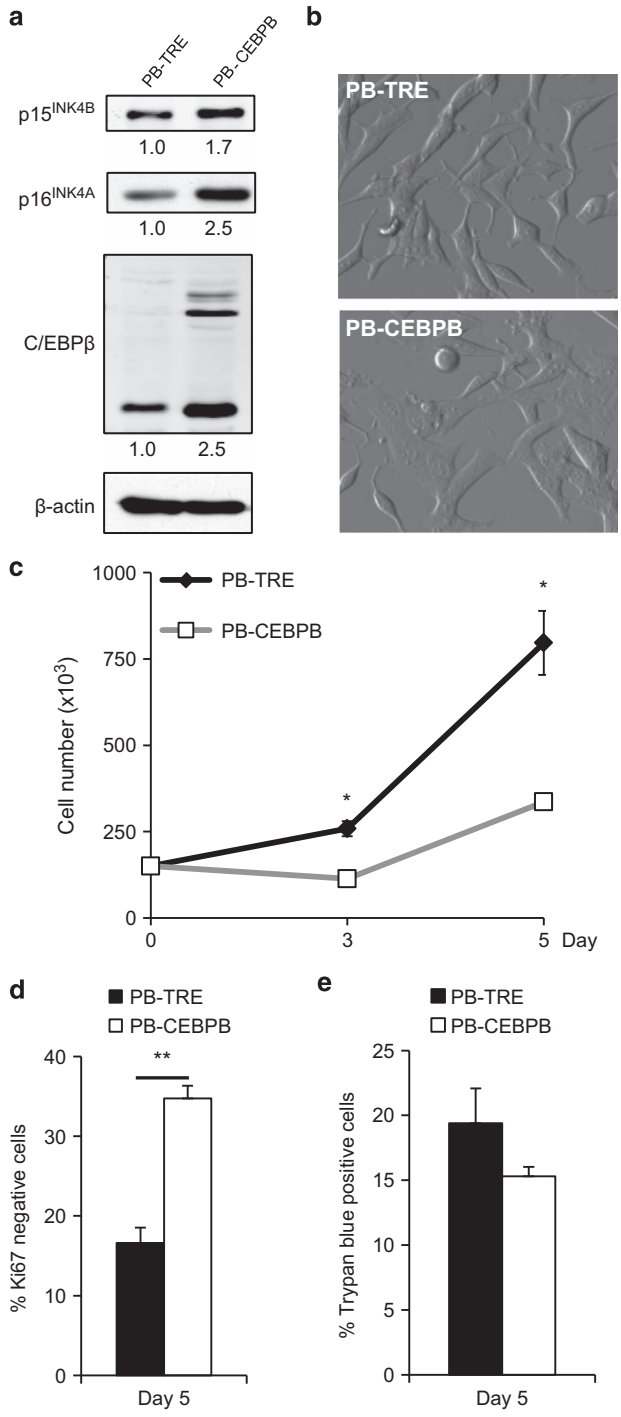


**Figure 4.** Ectopic expression of C/EBPβ suppresses LNCaP cell growth. (**a**) LNCaP PB-CEBPB and PB-TRE control cells were cultured for 5 days in 0.5 μg/ml doxycycline. Cell lysates were obtained and a representative western blot analysis for C/EBPβ, $p15^{INK4B}$ and $p16^{INK4A}$ is shown. Numbers below the blots indicate the relative band density. (**b**) Phase-contrast images of LNCaP PB-TRE and PB-CEBPB cells after 5 days in culture with 0.5 μg/ml doxycycline. (**c**) 1.5E5 LNCaP PB-CEBPB or PB-TRE cells were seeded in media containing 0.5 μg/ml doxycycline. Cells were enumerated on day 3 and 5. (**d**) Cells were cultured under similar conditions and the Ki67 expression was analyzed after 5 days of doxycycline treatment. (**e**) The number of dead cells on day 5 was assessed by trypan blue exclusion. All graphs represent the average of three experiments, error bars: s.e.m.; $^{*}P < 0.01$; $^{**}P < 0.02$.

Cellular senescence engenders a pro-survival phenotype. Given rapid induction of C/EBPβ in the absence of AR signaling and its role in directing senescence, we evaluated whether targeting C/EBPβ synergizes with anti-androgen agents or chemotherapy in killing PC cells. Culture of LNCaP shNTV and shCEBPB in ADM (Figure 8a) or exposing them to bicalutamide (Figure 8b) increased the number of dead cells relative to ARM cultures. However, there was no difference in cell viability between shNTV and shCEBPB cells (Figures 8a and b). However, after pre-culture in ADM, treatment with docetaxel or etoposide induced a significant 68% or 55% increase in cell death, respectively, in LNCaP cells harboring shCEBPB compared with shNTV (Figure 8c). Together, these data demonstrate that C/EBPβ promotes a pro-survival, drug-resistant phenotype during androgen deprivation.

## DISCUSSION

Accumulating evidence points to a strong connection between senescence and tumor progression.[23,24,36] Oncogene-induced senescence promotes the eventual emergence of subpopulations of aggressive, malignant cells and thus may be considered a tumor-promoting state. In PC, androgen-deprivation-induced senescence promotes the development of tumor progression and resistance to apoptosis,[5] fostering the emergence of cancer-initiating cells.[37] Increased numbers of senescent cells have been observed in tissue sections from tumors in patients that had been treated with neoadjuvant androgen deprivation therapy.[7] Also, cellular senescence induced by androgen deprivation dramatically increases reactive oxygen species and DNA double-strand breaks, and leads to the outgrowth of hormone-refractory populations in cultured LNCaP cells.[5] CRPC emerges as a result of multiple adaptations, including AR gene amplification, abnormal AR activation or enhanced steroidogenesis.[2] These changes are acquired and propagated as a result of selective pressure exerted on PC cells by an androgen-poor milieu while cells are protected by the androgen-deprivation-induced senescence state. Previous reports have suggested that androgen deprivation leads to a senescent state in both LNCaP and LAPC4 cells, but the mechanism by which these cells become senescent was not well described.[5–7] Here, we demonstrate that PC cells respond to androgen withdrawal by upregulating *CEBPB* transcription, that loss of C/EBPβ lead to a reduction in the number of senescent cells following androgen deprivation, and that ectopic expression of C/EBPβ induces the expression of senescent markers indicating that C/EBPβ plays a central role in cellular senescence induced by androgen deprivation, and that impeding the senescent response via inhibition of C/EBPβ expression keeps PC cells susceptible to chemotherapy, validating C/EBPβ as a therapeutic target in androgen-dependent PC.

Probing the Oncomine database revealed elevated expression of *CEBPB* in human CRPC samples. As CRPC is often characterized by active AR signaling, this finding may seemingly be at odds with our *in vitro* data showing that AR activity suppresses C/EBPβ expression. Several potential explanations may account for elevated C/EBPβ levels despite active AR signaling in CRPC. It has been demonstrated that there is substantial divergence in AR gene targets when comparing castrate-resistant to androgen-sensitive cells.[38] Further, androgen deprivation or castration resistance is associated with decreased AR occupancy on repressive DNA elements,[39] and the expression of many AR-repressed genes increases in castrate-resistant cells.[39] Finally, although castrate-resistant cells often exhibit increased levels of AR, AR signaling relative to androgen-sensitive PC cells may not increase because of diminished ligand levels. Therefore, C/EBPβ de-repression may persist in cells that have developed castration resistance.

Our data demonstrate that inhibition of AR leads to rapid upregulation of *CEBPB* RNA, loss of AR interaction with the *CEBPB*

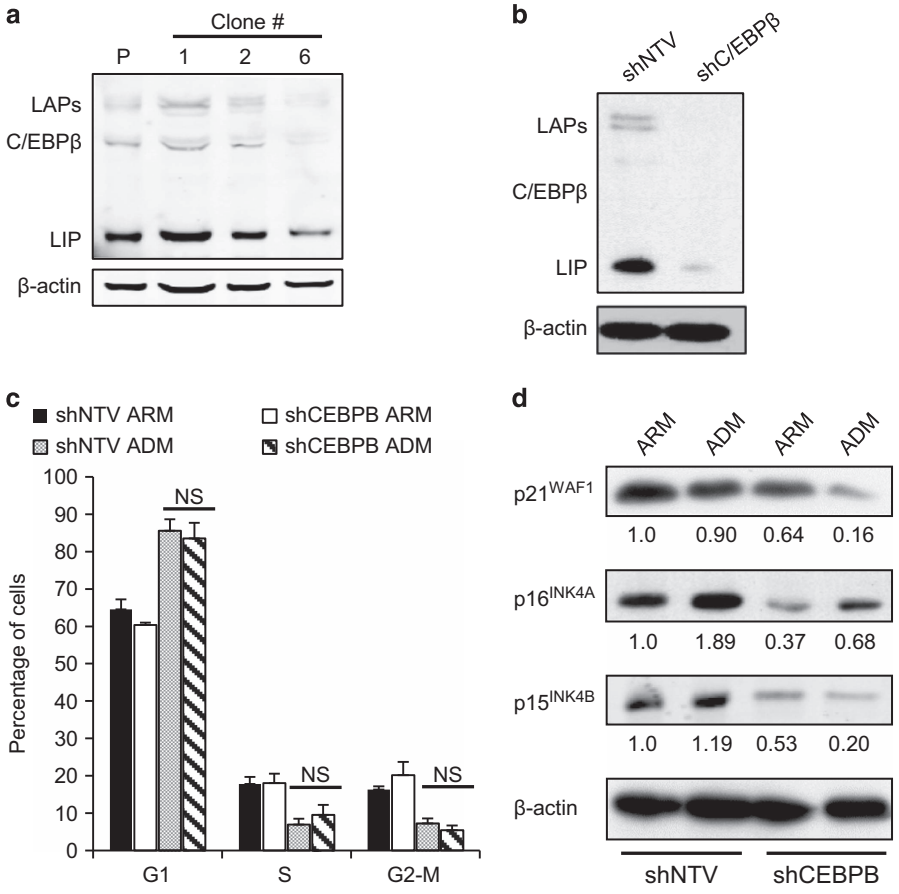


**Figure 5.** C/EBPβ is required for high expression levels of cell cycle inhibitors upon AR inhibition. (**a**) Total cellular proteins extracted from parental (P) LNCaP cells or individual clones transfected with TALEN-C/EBPβ were subjected to western blotting using C/EBPβ or actin antibodies. (**b**) LNCaP cells harboring an inducible shRNA against C/EBPβ (shC/EBPβ) or non-targeting vector (NTV) were cultured with doxycycline (200 ng/ml) for 48 h and protein extracts were subjected to immunoblotting with the indicated antibodies. (**c**) LNCaP cells harboring shCEBPB or non-targeting vector (shNTV) cultured with doxycycline (200 ng/ml) in ARM or ADM for 7 days, were stained with propidium iodide and DNA content was analyzed by flow cytometry. Mean distribution of cells in G1, S or G2/M from three experiments. (**d**) LNCaP cells expressing shNTV or shCEBPB were cultured in ARM or ADM for 7 days, and the expression of the indicated cell cycle regulators was assessed by western blotting.

promoter and increased promoter H3K acetylation. To our knowledge, this is the first demonstration that *CEBPB* is a direct AR transcriptional repressive target. This observation is consistent with previous reports showing that AR can inhibit gene expression through interaction with transcriptional co-repressors at proximal promoter regions.[40–42] Derepression of *CEBPB* occurs within 4 h of exposure to anti-androgens. Conversely, treatment with AR agonist R1881 results in diminished expression of C/EBPβ and suppression of the activity of a *CEBPB* promoter luciferase reporter. Accordingly, exposure to dihydrotestosterone leads to a decrease in activating AcH3 histone marks on the promoter. These findings indicate that AR suppresses *CEBPB* expression directly through regulation of the promoter. Examination of the *CEBPB* promoter sequence did not identify an androgen response element, suggesting indirect binding of AR. In other contexts, AR directly interacts with Sp1 to regulate gene expression in the absence of an androgen response element making Sp1 a potential mediator of AR regulation of *CEBPB*.[43,44]

Treatment of LNCaP cells with anti-androgens or culture in hormone-depleted media leads to G1 arrest and cellular senescence. PC cells require AR signaling for transition from G1 to S, and accordingly, we did not observe continued proliferation of cells that had been cultured in hormone-depleted media or with anti-androgens regardless of *CEBPB* knockdown. Androgen-deprivation-induced senescence had a profound long-lived effect on PC cell proliferation in the presence of normal C/EBPβ levels even after reintroduction of androgens, as previously observed.[6] We found that after androgen deprivation, C/EBPβ deficiency allowed LNCaP cells to resume proliferation when re-seeded in ARM. Thus, C/EBPβ plays an important role in the complete maintenance of senescent growth arrest induced by androgen deprivation.

Senescent cells develop unique secretory paracrine activities, conferring a pro-malignant microenvironment by secreting an array of cytokines and proinflammatory mediators.[24,45,46] Our results raise the possibility that C/EBPβ also promotes the expression of senescence-associated secretory genes such as IL-8 and IGFBP3 and the cell cycle inhibitors $p21^{WAF1}$ and $p15^{INK4B}$. IL-8 signaling has been shown to promote cell survival, angiogenesis and senescence in pre-clinical models of PC.[8,47,48] It activates the PI3K-AKT-mTOR pathway, which is critical for cell survival during androgen deprivation.[49] IGFBP3 is strongly upregulated following androgen deprivation and promotes tumor growth in a mouse PC model.[50,51] In PC patients, the percentage of cells positive for $p15^{INK4B}$ was shown to increase with tumor grade.[52–54] Expression of $p21^{WAF1}$ correlates with a worsened prognosis both before and after androgen deprivation therapy, and *in vitro* studies of $p21^{WAF1}$ have shown that it suppresses

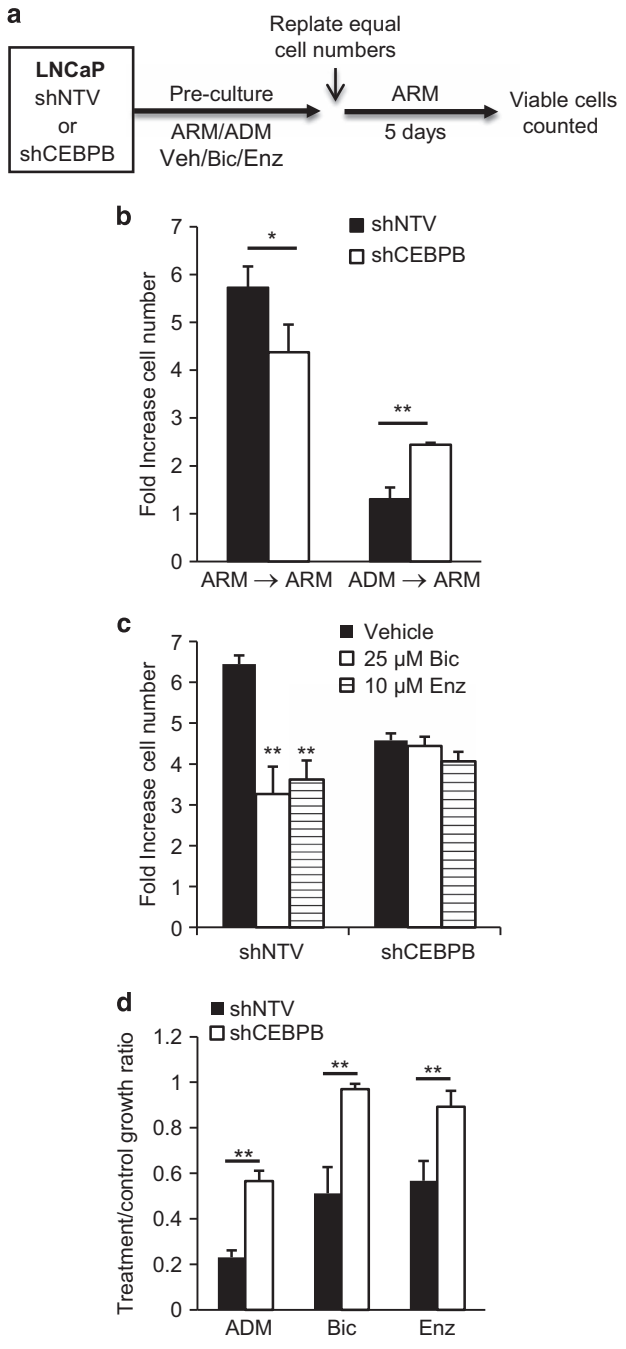


**Figure 6.** C/EBPβ is required for complete maintenance of growth arrest induced by AR inhibition in LNCaP cells. (**a**) LNCaP cells expressing shCEBPB or shNTV were pre-cultured in doxycycline under the indicated conditions or in ARM control media, 1.5E5 cells from each pre-culture condition were replated and enumerated after 5 days, as depicted in the diagram. (**b**) After 7 days pre-culture in ARM or ADM, equal number of shCEBPB or shNTV LNCaP cells were re-plated in ARM in a 6-well dish and counted 5 days later. The average fold increase in cell number is shown. (**c**) Similarly, cells were pre-cultured for 4 days with bicalutamide (Bic), enzalutamide (Enz) or vehicle control and doxycycline, re-plated in ARM and enumerated after 5 days as described above. (**d**) The relative growth ratio of cells pre-cultured in ADM, bicalutamide (Bic) or enzalutamide (Enz) over cells cultured in ARM control conditions. Bar graphs represent the mean of at least three experiments ± s.e.m.; $^{*}P < 0.05$; $^{**}P < 0.005$.

apoptotic response to chemotherapy.[55–57] These findings are consistent with our results showing that C/EBPβ-deficient cells, with decreased $p21^{WAF1}$ and $p15^{INK4B}$ levels, were more sensitive to chemotherapy post androgen withdrawal. Increased $p16^{INK4A}$ expression is not essential to androgen-induced senescence,[5] and we see suppression of $p16^{INK4A}$ in C/EBPβ-deficient LNCaP cells. Overall, C/EBPβ promotes PC senescence and thereby potentially chemo-resistance and progression to castration-resistant growth through multiple transcriptional targets during androgen deprivation therapy (Figure 8d).

Our delineation of *CEBPB* upregulation in human hormone-refractory PC further support the concept that C/EBPβ-dependent induction of senescence during androgen blockade promotes castration-resistant progression by providing the opportunity to respond to the selective pressure of anti-androgen therapy. Importantly, these data indicate the potential utility of targeting C/EBPβ in combination with androgen deprivation for novel PC therapy.

## MATERIALS AND METHODS

### Cell lines and plasmids

LNCaP cells were maintained in RPMI media without phenol red with 10% heat-inactivated FBS (HI-FBS) (Hyclone Laboratories, Logan, UT, USA) supplemented with penicilin/streptomycin. LAPC4 cells were maintained in Iscove's modified Dulbecco's media (IMDM) supplemented with 15% HI-FBS, 1 nM dihydrotestosterone and penicillin/streptomycin. DU145 cells were maintained in RPMI with 10% HI-FBS, and 293T cells were cultured in Dulbecco modified Eagle medium with 10% HI-FBS. Cells were grown in a humidified incubator maintained at 37 °C with 5% $CO_2$. Cells were split 1:4 and were used until passage 40. Cells transduced with pTRIPZ-shRNA or transfected with pPB-TRE-Puro were maintained in tetracycline-screened FBS (Hyclone Laboratories). For androgen deprivation, cells were cultured in phenol red-free media supplemented with 10% charcoal-stripped FBS (Hyclone Laboratories). AR was blocked using enazlutamide (Selleckchem, Houston, TX, USA) or bicalutamide (Sigma-Aldrich, St Louis, MO, USA).

pTRIPZ-shRNA (Open Biosystems, Lafayette, CO, USA) lentiviral vectors were generated as described[58] and stably transduced cells were selected with puromycin (2 μg/ml) after 48 h. Expression of shRNA was induced by treating cells with 200 ng/ml doxycycline, replaced every 48 h and confirmed by fluorescence microscopy detection of RFP.

The pPB-TRE-Puro plasmid (kindly provided by Jolene Ooi and Pentao Liu) contains a multiple cloning site downstream of a TRE element, and a CAG promoter upstream of rtTA, IRES and apuromycin resistance gene. The mouse *Cebpb* ORF including the 3′UTR (1–1400) was ligated as *BamH*I/*Not*I fragment into the *Bgl*II/*Not*I-digested pPB-TRE-Puro plasmid[29] to generate the pPB-CEBPB which was confirmed by sequencing. Stable PB-TRE and PB-CEBPB cell lines were generated by transfecting equal parts pCMV-hyperpiggybase and piggybac vectors by lipofection.

### Western blotting

Protein samples from whole-cell lysates and nuclear extracts were prepared and subjected to western blotting as previously described.[59] After blocking with Odyssey blocking buffer (LI-COR Bioscience, Lincoln, NE, USA), membranes were incubated overnight with the following primary antibodies: $p15^{INK4B}$ (M-20), C/EBPβ (C-19), AR (N-20) (Santa Cruz Biotechnology, Dallas, TX, USA), β-actin (AC15) (Sigma-Aldrich). For target protein detection, membranes were incubated with secondary antibodies, goat anti-mouse Alexafluor 670 (Life Technologies, Carlsbad, CA, USA) or goat anti-rabbit antiserum (LI-COR) and imaged on a Li-Cor Odyssey Fc infrared imaging system.

### Quantitative real-time PCR

Total RNA was isolated from cells and first strand cDNA was synthesized as previously described.[58] β-actin transcript was used as a reference to normalize samples and relative expression was calculated as described.[58] Each sample was assayed in triplicates and each experiment was repeated at least three times. Oligonucleotides were custom ordered from Sigma-Aldrich, and their sequences are presented in Table 1.

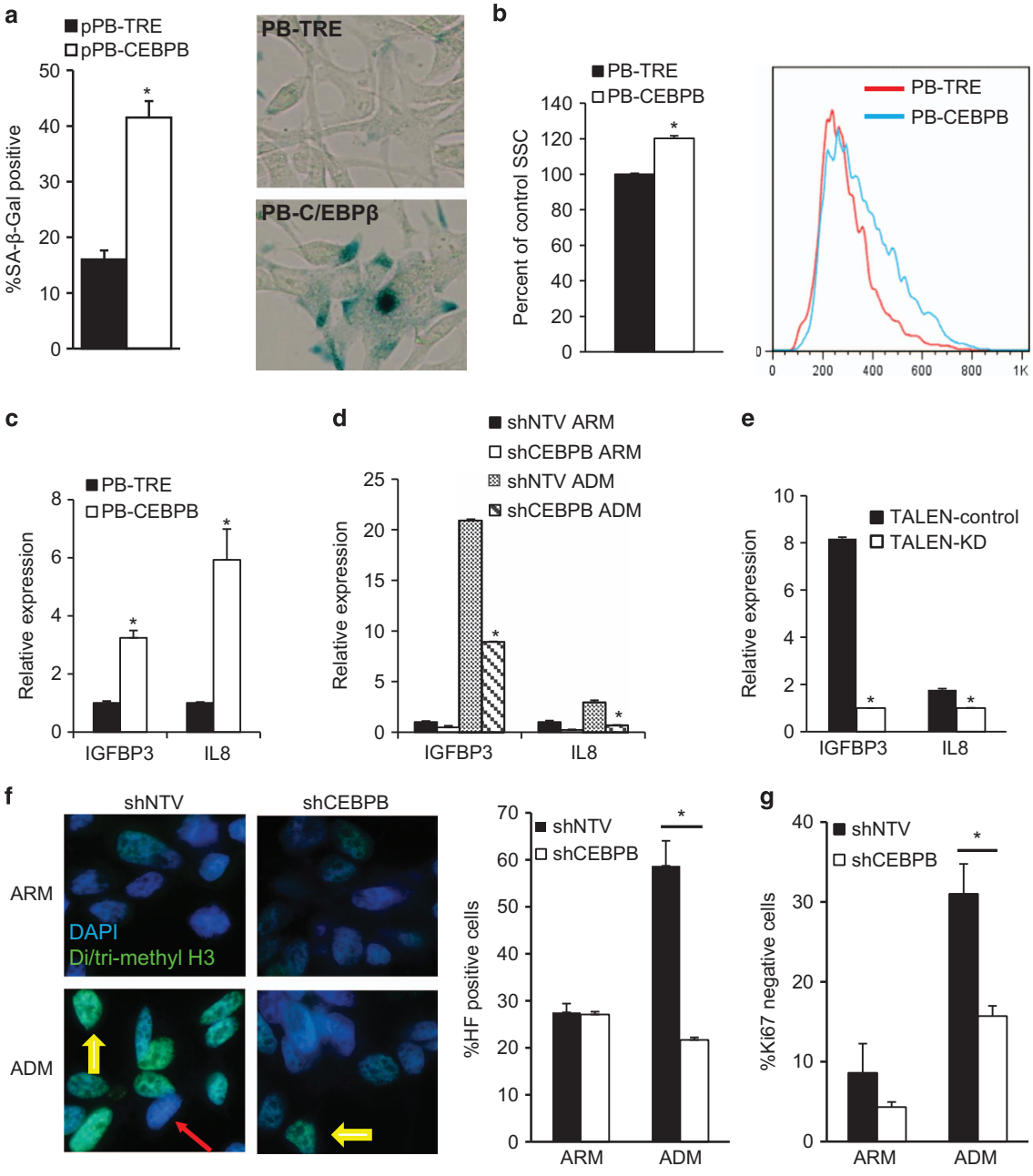


**Figure 7.** C/EBPβ is required for androgen-deprivation-induced senescence. (**a**) The percent of SA-β-gal-positive PB-TRE or PB-CEBPB cells was quantified after 5 days of culture in 0.5 μg/ml doxycycline. An average from three experiments (left) and representative images are shown (right). (**b**) Quantification of cell granularity by the median side scatter value of G1 PB-TRE and PB-CEBPB cells 5 days after seeding in doxycycline. A representative histogram is shown on the right. (**c**) IL8 and IGFBP3 expression were quantified by qRT–PCR in PB-TRE and PB-CEBPB cells cultured under the above conditions. Conversely, total cellular RNA was extracted from shNTV or shCEBPB (**d**), or TALEN-control or TALEN-KD (**e**) LNCaP cells after 7 days in culture in ARM or ADM and transcript levels of IL8 and IGFBP3 were assessed by qRT–PCR. (**f**) Expression of an shRNA against *CEBPB* or non-targeting vector (shNTV) control were induced by doxycycline in LNCaP cells cultured in ARM or ADM. After 7 days, cells were stained for senescence-associated heterochromatin foci (HF) (yellow arrows). Red arrow points to HF-negative cells. Representative photomicrographs are shown (left, ×600 magnification). The average and s.e. of three independent counts is shown (right panel). *$P < 0.001$. (**g**) Ki67 was quantified using flow cytometry in shNTV or shCEBPB LNCaP cells cultured in ARM or ADM for 7 days and the average proportion of Ki67 negative cells from three experiments is presented. *$P < 0.01$.

### Luciferase reporter assays

Using PCR, a DNA fragment from −888 to +64 base pairs (bp) relative to the initiation of transcription (Ensembl database) of the human *CEBPB* promoter was cloned as a *Bam*HI/*Mlu*I fragment into *Bgl*II/*Mlu*I-digested pGL3-luciferase reporter vector (Promega, Madison, WI, USA) to generate CEBPB-Luc that was confirmed by Sanger sequencing. LNCaP or DU145 cells were seeded in 6-well plates and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Each well was co-transfected with 1.4 μg of the reporter plasmid and 10 ng of CMV-β-galactosidase as an internal control. Twenty four hours after transfection, cells were treated with vehicle (0.01% ethanol) or the androgen agonist R1881 (Sigma-Aldrich) and after additional 24 h, cells were lysed (Reporter Lysis Buffer, Promega) and assayed for luciferase and β-galactosidase activity. Fold activation relative to the vehicle-treated cells after correction for β-galactosidase activity was determined. Lysates from non-transfected cells were used as baseline.

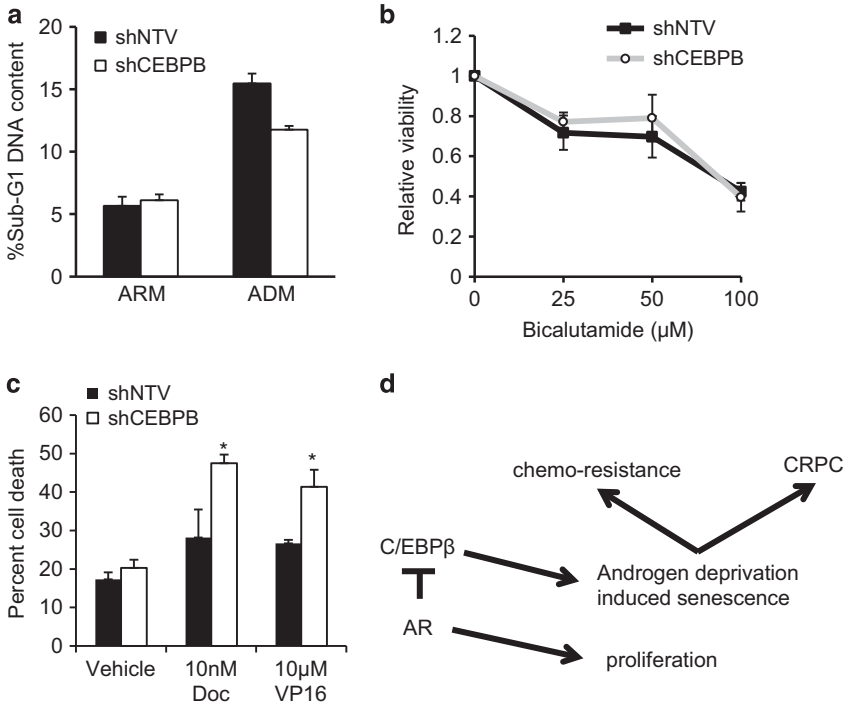


**Figure 8.** C/EBPβ protects from chemotherapy following androgen deprivation. (**a**) LNCaP cells were cultured for 1 week in ARM or ADM, and cell death was evaluated flow cytometric analysis of sub-G1 DNA content. (**b**) LNCaP shNTV or shCEBPB lines cultured in ADM were exposed to bicalutamide at the indicated dose for 48 h and relative cell viability was evaluated by the WST-1 assay. The average cell viability from three independent experiments is shown. (**c**) LNCaP lines harboring shNTV or shCEBPB were cultured for 7 days in ADM, re-plated in ARM, and treated with vehicle control, 10 nM docetaxel (Doc), or 10 μM etoposide (VP16) for 48 h. Cell survival was assessed by trypan blue exclusion and the average percentage of dead cells from three independent experiments is shown. (**d**) Model of the regulation of C/EBPβ expression by AR. Upon AR inhibition, C/EBPβ expression will increase to promote senescence, chemoresistence and emergence of CRPC.

**Table 1.** Primers used for ChIP and RNA analysis

| *Gene* | *Forward sequence* | *Reverse sequence* |
|---|---|---|
| *ChIP* | | |
| CEBPB proximal | GGCCGCCCTTATAAATAACC | TATTAGTGAGGGGGCTGGTG |
| CEBPB upstream | ATAATGGTGGCTGGCGATAG | CCTTCCTCACTGCAAAATGG |
| *Real-time PCR* | | |
| TMPRSS2 | AATCCCCATCCGGGACAGT | AGGAGTCGCACTCTATCCCA |
| PSA | GCAGCATTGAACCAGAGGAG | AGAACTGGGGAGGCTTGAGT |
| ACTB | GACCTGGCTGGCCGGGACCT | GGCCATCTCTTGCTCGAAGT |
| GAPDH | CCACCCATGGCAAATTCC | GATGGGATTTCCATTGATGACA |
| CEBPB | AAACTCTCTGCTTCTCCCTCTGC | CTGACAGTTACACGTGGGTTGC |
| IL8 | TCTGGCAACCCTAGTCTGCT | GCTTCCACATGTCCTCACAA |
| BDNF | GCGTGTGTGACAGTATTAGT | CTGGGTAGTTCGGCACTGGG |
| CGA | GCGGTGGAAGAGCCATCAT | TCTGTGGCTTCACCACTTTTCTC |
| IGFBP3 | GCCAGCGCTACAAAGTTGAC | ATGTGTACACCCCTGGGACT |
| P16 INK4A | CCGAATAGTTACGGTCGGAGG | CACCAGCGTGTCCAGGAAG |
| P15 INK4B | GAGGCGCGCGATCCAG | CACCAGCGTGTCCAGGAAG |
| P21 CIP1/WAF1 | ACTCTCAGGGTCGAAAACGG | GATGTAGAGCGGGCCTTTGA |

Abbreviation: ChIP, chromatin immunoprecipitation.

## TALEN construction and CEBPB gene editing

TALEN DNA constructs targeting the human *CEBPB* ORF were constructed using the Golden Gate Talen assembly kit (Addgene, Cambridge, MA, USA).[30] Targeting sequences were designed using the Cornell University TAL Effector Nucleotide Targeter 2.0 web-based software. Golden Gate assembly of the repeat-variable di-residue sequence was performed according to the manufacturer's instructions, and the completed TALEN pairs were ligated into the pTAL3 vector. The complete TALEN ORF including the repeat-variable and FokI domains was excised using *Xho*I and *Apa*I restriction endonuceases and ligated into the pcDNA3.1(+) vector. LNCaP cells cotransfected with TALEN expression vectors targeting *CEBPB* were seeded in 96-well dishes, and individual clones were screened for C/EBPβ expression by western blotting.

## DNA content analysis and flow cytometry

Cell cycle analysis by DNA content was performed as previously described.[60] Ki67-expressing cells were identified by flow cytometry using APC-anti-Ki67 antibody (BioLegend, San Diego, CA, USA). Flow cytometry analysis was performed using a BD FACSCalibur machine (BD Biosciences, San Jose, CA, USA). Sub-cellular debris and dead cells were gated out and singlet discrimination was performed by gating on FL2-A and FL2-W channels and data were interpreted using FloJo Cytometric Analytical software (TreeStar, Ashland, OR, USA).

## Chromatin immunoprecipitation

5E6 LNCaP cells were used in each chromatin immunoprecipitation reaction as previously described,[58] using antibodies against C/EBPβ, AR, rabbit IgG (Santa Cruz Biotechnology) or acetylated histone H3 (06–599) (Millipore, Billerica, MA, USA). DNA fragments corresponding to the promoters of interest were detected by PCR using the primers presented in Table 1.

## Cell viability and proliferation assays

Viability was determined using the WST-1 assay (Roche, Indianapolis, IN, USA). Briefly, cells were seeded into 96-well plates, allowed to adhere for 48 h and then treated for an additional 48 h. WST-1 reagent (Roche) was directly added to the wells, and after incubation, absorbance was read at 450 nm using a Bio-Rad (Hercules, CA, USA) Microplate Reader Model 680. The 670 nm reference absorbance and readings from blank wells containing only cell culture media and DMSO (vehicle) were subtracted from experimental wells. Relative viability was determined by dividing absorbance readings from vehicle-treated wells. For cell proliferation, LNCaP cells were grown for 7 days in androgen depleted media. Cells were trypsinized, stained with Trypan blue dye and viable cells were enumerated using a hemocytometer. 1.5E5 cells per well were seeded into 6-well plates in ARM. After 5 days, cells were similarly enumerated.

### SA-β-gal chromogenic assay

SA-β-gal-positive cells were stained using the chromogenic assay as described.[61] Five random fields of view were imaged on a Leica E600 (Leica, Buffalo Grove, IL, USA) microscope by brightfield microscopy at ×200 magnification. Positive cells were identified as those containing blue precipitate throughout the cytoplasm.

### Immunofluorescent staining and heterochromatin foci quantification

LNCaP cells were seeded onto poly-D-lysine-coated glass coverslips and following treatment, were fixed in 4% paraformaldehyde. Cells were washed and incubated in permeabilization buffer (TBS, 2% BSA, 0.5% Triton-X 100, 0.1% sodium azide). After blocking, cells were incubated with anti-di/trimethyl H3K9 (1:250, Cell Signaling Technology, Danvers, MA, USA), washed and incubated with goat anti-mouse-Alexafluor 488-conjugated secondary antibody (Life Technologies). Cells were washed and mounted on glass slides for analysis by fluorescence microscopy. Heterochromatin foci were imaged on a Leica E800 fluorescence microscope with a CCD camera and imaged at ×630 magnification with an oil immersion objective. Fluorescent micrographs of heterochromatin foci from six to seven random fields of view were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA). Intensely stained nuclei with multiple fluorescent foci that colocalized with DAPI staining were counted as positive cells containing heterochromatin foci.

### Statistical analysis

Statistical comparison of two groups of samples was conducted using the Student's *t*-test. Comparisons of multiple groups of samples was performed using the analysis of variance followed by multiple comparisons with the Student's *t*-test and the Holm-Bonferonni Correction ($\alpha/(n-k+1)$, where $n$ = number of comparisons and $k$ = rank of *P*-value).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

DJB designed and performed the research, analyzed data and wrote the manuscript; JZ and TB performed the research; SRD designed the research and analyzed data; ADF designed the research, analyzed data and wrote the manuscript; IP-P designed and performed the research, analyzed data and wrote the manuscript.

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